Assessing variability and comparing short-term biomarkers of styrene exposure using a repeated measurements approach

S. Fustinoni a,∗, P. Manini b, L. Campo a, G. De Palma c, R. Andreoli b, A. Mutti b, P.A. Bertazzi a, S.M. Rappaport d

a Department of Occupational and Environmental Health, University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico,
Mangiagalli e Regina Elena, via S. Barnaba, 8 20122 Milan, Italy
b Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, Parma, Italy
c Department of Experimental and Applied Medicine, Unit of Occupational Hygiene, Toxicology and Prevention, University of Brescia, Brescia, Italy
d Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA, USA

A R T I C L E   I N F O

Article history:
Available online 11 February 2009

Keywords:
Variability
Styrene
Biomarkers

A B S T R A C T

The aim of this work is to compare several short-term biomarkers of styrene exposure, namely urinary styrene (StyU), mercapturic acids (M1 + M2), mandelic acid (MA), phenylglyoxylic acid (PGA), phenylglycine (PHG), and 4-vinylphenol conjugates (VP), for use as biomarkers of exposure in epidemiologic studies. A repeated measurements protocol (typically 4 measurements per worker over 6 weeks) was applied to measure airborne styrene (StyA) and urinary biomarkers in 10 varnish and 8 fiberglass reinforced plastic workers. Estimated geometric mean personal exposures to StyA were 2.96 mg/m³ in varnish workers and 15.7 mg/m³ in plastic workers. The corresponding levels of StyU, M1 + M2, MA, PGA, MA + PGA, PHG and VP were 5.13 µg/L, 0.111, 38.2, 22.7, 62.6, 0.978, and 3.97 mg/g creatinine in varnish workers and 8.38 µg/L, 0.303, 146, 83.4, 232, 2.85 and 3.97 mg/g creatinine in plastic workers. Within-worker (σ²w) and between-worker (σ²b) variance components were estimated from the log-transformed data as were the corresponding fold ranges containing 95% of the respective lognormal distributions of daily levels (wR0.95) and subject-specific mean levels (bR0.95). Estimates of wR0.95 (range: 4–26) were generally smaller than those of bR0.95 (range: 5–790) for both environmental and biological markers; this indicates that exposures varied much more between workers than within workers in these groups. Since attenuation bias in an estimated exposure–response relationship increases with the variance ratio λ = σ²b/σ²w, we estimated values of λ for all exposure measures in our study. Values of λ were typically much less than one (median = 0.220) and ranged from 0.089 for M1 + M2 in plastic workers to 1.38 for PHG in varnish workers. Since values of λ were 0.147 and 0.271 for StyA in varnish workers and plastic workers, respectively, compared to 0.178 and 0.210 for MA in the same groups, our results suggest that either air measurements or conventional biomarker measurements (urinary MA) would be comparable surrogates for individual exposures in epidemiologic studies.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Styrene is an important industrial chemical primarily used in the production of plastics (Miller et al., 1994; IARC, 1994). Due to its toxicological properties styrene has been classified as a possible carcinogen to humans (group 2B) (IARC, 1994). Its occupational exposure is regulated in many countries with an airborne concentration of 85 mg/m³ (20 ppm) currently recommended as an exposure limit by both the American Conference of Governmental Industrial Hygienists (ACGIH, 2007) and the Deutsche Forshungsgemeinschaft (DFG, 2007).

The metabolites mandelic acid (MA) and/or phenylglyoxylic acid (PGA) in end-shift urine have generally been the biomarkers of choice for styrene exposure [Biological Exposure Index = 400 mg (MA + PGA)/g creatinine (ACGIH, 2007) and Biological Tolerance Value = 600 mg (MA + PGA)/g creatinine (DFG, 2007)], although blood styrene in end-shift samples has also been recommended [Biological Exposure Index = 0.2 mg/L (ACGIH, 2007)]. Recently, other metabolites of styrene, including a mixture of diastereomeric mercapturic acids, [(RR)- and (S,R)-N-acetyl-S-(1-phenyl-2-hydroxyethyl)-l-cysteine and (RR)- and (S,R)-N-acetyl-S-(2-phenyl-2-hydroxyethyl)-l-cysteine] designated as M1 + M2, 4-vinylphenol (VP), excreted as a glucuronide and sulfate conjugates, phenylglycine (PHG) and urinary styrene (StyU) have also been proposed as biomarkers of styrene exposure (Manini et al., 2003; Haufroid et al., 2001; De Palma et al., 2001; Ghittori et al., 1987).
It has been shown that air and biological measures of chemical exposure vary both within and between workers in a given observational group (Liljelind et al., 2003; Kromhout et al., 1993). Since these sources of variability have profound implications on our ability to make valid and precise inferences about the levels of occupational exposure in investigations of either health effects or for the control of occupational hazards, it is important that the within- and between-worker variance components, designated as \( \sigma^2_{wY} \) and \( \sigma^2_{bY} \), respectively, be characterized (Rappaport and Kupper, 2008). These variance components can be estimated by applying analysis of variance (ANOVA) or linear mixed-effects models to data containing repeated measurements of air and/or biomarker measurements from representative workers in each observational group. Of particular interest is the variance ratio \( R = \sigma^2_{wY}/\sigma^2_{bY} \) which provides an index of the potential for a given exposure surrogate, i.e., a particular type of air measurement or biomarker, to introduce attenuation bias into an exposure–response relationship (Lin et al., 2005; Rappaport and Kupper, 2008). Thus, by selecting from the many possible measures of exposure to styrene, i.e., StyA and the various urinary biomarkers, the exposure measure with the smallest variance ratio \( R \) should minimize the potential bias in an exposure–response relationship due to exposure measurement error.

In a recently published study we focused on an integrated approach to investigate urinary analytes and haemoglobin and albumin adducts as biomarkers of exposure to airborne styrene (StyA) and styrene-(7,8)-oxide (StyOX), and to evaluate the influence of smoking habit and genetic polymorphism of metabolic enzymes GSTM1 and GSTTI on these biomarkers (Fustinoni et al., 2008). To accomplish this, we employed a repeated measurement sampling design in which StyA, as well as urine specimens, were repeatedly collected over a 6-week period in groups of varnish workers and reinforced plastic workers exposed to styrene. In the present study a subset of these data were further analyzed to estimate within-worker and between-worker variance components and the corresponding values of \( R \) for several potential surrogates of styrene exposure, namely, StyU, M1 + M2, MA, PGA, PHG, and VP.

2. Materials and methods

2.1. Study population, design, air and biological sampling

Two groups of male subjects employed in a varnish production plant and in a fiberglass reinforced plastic industry located in Northern Italy were involved in the study. The study was conducted during the periodical health surveillance program and subjects were recruited with the help of the plant’s occupational physician. They were informed about the aims and the protocol of the study and provided written consent to be included as human subjects.

All the subjects working in the production department entered the study for a total of 13 varnish workers and 8 plastic workers (Fustinoni et al., 2008). The study design aimed to collect 4 repeated personal air samples and end-shift urine samples over a period of 6 consecutive weeks. Since 3 varnish workers were absent during most of investigated days, they were excluded from the present analysis that finally included 10 varnish workers and 8 plastic workers.

Information about work activities, demographic and lifestyle factors, and medical histories were obtained using a questionnaire administered by an occupational physician (Table 1). Subjects were healthy, and none of them had history of liver or other chronic diseases.

Varnish workers were involved in the production of styrene-containing varnishes as a continue process, while fiberglass reinforced plastic workers were involved in boat production using open mould in a discontinuous process. In both activities individual tasks were the same all over the investigated period. Workers in the same plant were investigated on the same day, and for each day information on job task was collected.

Information about job title, sampling day, frequency of sampling, number of sampling for worker and interval between the repeated measurements is reported in Table 1.

Finally, following this design 38 air and urine samples from the varnish workers and 30 from the plastic workers for the determination of StyA and StyU, M1 + M2, MA, PGA, PHG and VP were collected.

### Table 1

<table>
<thead>
<tr>
<th>Varnish workers</th>
<th>Fiberglass reinforced plastic workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>10</td>
</tr>
<tr>
<td>Age’</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Smokers [%]</td>
<td>38</td>
</tr>
<tr>
<td>Number of cigarette/day’</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>Job title</td>
<td>6 producers, 1 warehouse man, 2 quality control technicians, 1 supervisor</td>
</tr>
<tr>
<td>Sampling day</td>
<td>Wednesday/Thursday</td>
</tr>
<tr>
<td>Number of samplings</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>0.7</td>
</tr>
<tr>
<td>Interval between the repeated measurement’ (days)</td>
<td>12 ± 3.6</td>
</tr>
</tbody>
</table>

*’ = mean ± SD

2.2. Analysis of air and biological samples

2.2.1. Airborne styrene

The air concentrations of StyA were determined as described by Tomerino-Velez et al., 2000. The detection limit was 0.3 mg/m³.

2.2.2. Unmetabolized styrene in urine

Levels of StyU were determined by headspace solid-phase microextraction (SPME) followed by GC–MS analysis as previously described by Fustinoni et al., 2008. The detection limit was 0.2 μg/L.

2.2.3. Styrene metabolites in urine

Urinary metabolites were assayed by liquid chromatography with tandem mass spectrometry (LC–MS/MS), as previously described (Manini et al., 2002). Limits of detection were 0.1 mg/L for both MA and PGA, 0.01 mg/L for PHG and 0.0004 mg/L for each mercapturic acid, 0.015 mg/L for VP-G and 0.005 mg/L for VP-S. Concentrations of metabolites in urine samples were expressed as a function of creatinine concentration, measured by the method of Jaffe. Creatinine levels ranging between 0.3 and 3.0 g/L were considered acceptable (WHO, 1996).

2.3. Estimation of variance components

Within- and between-worker variance components (\( \sigma^2_{wY} \) and \( \sigma^2_{bY} \)) were estimated by applying a one-way random effects (ANOVA) model to the data from each group of workers separately. Analyses were applied after natural logarithmic transformation of air or urinary measurements to achieve approximate normality and homogeneity of variance (Rappaport and Kupper, 2008). The following model was used:

\[
Y_{ij} = \ln(X_{ij}) = \mu + w_i + b_j + e_{ij}
\]

for \( i = 1, \ldots, k \) persons and \( j = 1, \ldots, n \) days, where \( Y_{ij} \) represents the exposure level or the urinary metabolite for the \( i \)th person on the \( j \)th day. The mean and variance of \( Y_{ij} \) are designated as \( \mu \) and \( \sigma^2_{Y} \), respectively. Under Model (1), \( w_i \) represents the true fixed mean (logged) exposure or biomarker level for the group; \( b_j \) represents the random effect of the \( j \)th person; \( e_{ij} \) represents the random deviation of the observed logged exposure level \( Y_{ij} \) on the \( j \)th day for person \( i \) from the subject-specific mean (logged) level \( \mu \). It is assumed that \( b_j \) and \( e_{ij} \) are mutually independent and normally distributed random variables, with means of zero and variance \( \sigma^2_{b} \) and \( \sigma^2_{e} \), respectively. Thus, the total variability in the logged exposure levels experienced by a group is given by \( \sigma^2_{Y} = \sigma^2_{w} + \sigma^2_{b} + \sigma^2_{e} \). Also, \( Y_{ij} = \ln(X_{ij}) \) is normally distributed with mean \( \mu + w_i \) and variance \( \sigma^2_{w} \).

StyA and urinary biomarker data from the 10 varnish workers and the 8 plastic workers, with 4 nominal measurements per worker were fit in Model (1). Following Rappaport (1991), scale-independent measures of within-worker and between-worker variability, i.e., \( \sqrt{R_{w}} = \exp(3.92w_{\text{ys}}) \) and \( \sqrt{R_{b}} = \exp(3.92b_{\text{ys}}) \), were also estimated. Note that \( w_{\text{ys}} \) represents the fold range containing 95% of the \( X \) values for the \( i \)th worker and \( b_{\text{ys}} \) represents the fold range containing 95% of the subject-specific mean exposure levels for the group of workers.

2.4. Bias in estimating exposure–response relationship

The variance ratio \( R = \sigma^2_{wY}/\sigma^2_{bY} \) can be used to evaluate the potential that measurement error in a given exposure metric will lead to attenuation bias in an exposure–response relationship (Rappaport, 1991; Rappaport and Kupper, 2004; Liljelind et al., 2003; Lin et al., 2005). Assume an individual-based study design where the health outcome and exposure levels are measured in each member of a...
sample from an observational group of workers and where the relationship between the logarithms of these variables is linear. Let \( \theta_{true} \) represent the true slope of the log-response log-exposure relationship for a population of workers. Assuming that each worker in a sample from the population has \( n \) randomly collected exposure measurements (either air or biomarker), then the estimated mean of the logged exposure values, i.e., \( Y_i = (1/n) \sum_{j=1}^{n} y_{ij} \), can be used as a surrogate for the true (logged) individual exposure \( \mu_{Yi} \). Let \( \delta' \) represent the least-squares estimate of \( \theta_{true} \), based upon the observed health-response and the observed measure of exposure (i.e., \( Y_i \)) for each worker in the sample. Since the relationship between \( \delta' \) and \( \theta_{true} \) is given by the following expression:

\[
\delta' = \frac{\theta_{true}}{1 + \lambda/n},
\]

we see that \( \delta' \) is suppressed, or attenuated, relative to \( \theta_{true} \) and that the attenuation increases with increasing \( \lambda \) and decreases with increasing \( n \). Thus, for a given \( n \), the exposure surrogate (air or biomarker) with the smallest variance ratio \( \lambda \) should be the least biasing measure of exposure for investigating an exposure–response relationship (Lin et al., 2005).

### 2.5. Statistical analyses

The statistical analyses were carried out using the SPSS 15.0 (SPSS Inc. Chicago, IL, USA) and Excel (Microsoft, USA) work packages.

### 3. Results

Raw data for StyA and selected urinary metabolites are summarized in Fig. 1 which shows scatter plots of the air and biomarker levels from the varnish workers and plastics workers. For each subject all individual measurements (observations of \( Y_{ij} \)) are shown as well as the estimated subject-specific mean values (values of \( \mu_{Yi} \)). The comparison of scatter plots in Fig. 1 reveals similar patterns for the different measures of exposure in that the within-subject variability is small compared to the between-subject variability.

The two subjects with the highest styrene exposure were laminators of the plastic industry, with geometric mean (GM) levels \([i.e., \text{GM} = \exp(\mu_{Yi})]\) of 88 and 93 mg/m\(^3\), both exceeding the occupational exposure limit of 85 mg/m\(^3\). These subjects consistently had the highest values for all biomarkers. For example, \( \text{GM} \) of MA + PGA was 780 and 765 mg/g creatinine, which exceeded the Biological Exposure Index of 400 mg/g creatinine (ACGIH, 2007) and the Biological Tolerance Value of 600 mg/g creatinine (DFC).

The estimated group mean exposure levels (\( \mu_{Y} \)) and group GM values (i.e., \( \text{GM} = \exp(\mu_{Y}) \)) are given in Table 2 along with the within- and between-worker variance components and corresponding \( R_{0.95} \) values and variance ratios. For the varnish workers the estimated GM for StyA was 2.96 mg/m\(^3\), while for the plastic workers it was 15.7 mg/m\(^3\). Biomarker levels clearly reflected StyA levels where the estimated GM values for StyU, M1 + M2, MA, PGA, MA + PGA, PHG and VP were 5.13 \( \mu \)g/L, 0.111, 38.2, 22.7, 62.6, 0.978, and 1.38 mg/g creatinine in varnish workers, and 8.38 \( \mu \)g/L, 0.303, 146, 83.4, 232, 2.85 and 3.97 mg/g creatinine in plastic workers. In both groups MA was the most abundant metabolite and the sum of M1 + M2 was the least abundant.

The estimates of \( \sigma_{Y}^2 \) and \( \sigma_{\beta}^2 \) confirm the visual observations from Fig. 1; that is, between-subject variability tends to be larger than within-subject variability in the majority of cases and for both varnish and plastic workers. This finding is reinforced by the fold ranges, where \( gR_{0.95} \) ranged between 5 and 790 and \( wR_{0.95} \) ranged between 4 and 26. This dominance of between-worker variability resulted in variance ratios \( \lambda \) smaller than one in virtually all cases; indeed, StyU and PHG in varnish workers had values of \( \lambda \) greater than 1.

### 4. Discussion

In this study the within-subject and between-subject variance components of a panel of environmental and biological markers of styrene exposure were compared in two groups of workers exposed to styrene in different settings. We found that within-worker variability was typically much smaller than between-worker variability for the majority of exposure metrics. This yielded small variance ratios \( \lambda \) suggesting that most of the exposure metrics should provide relatively unbiased measures of exposure for epidemiologic studies. This finding was somewhat surprising because all the biomarkers investigated, i.e., urinary styrene and metabolites, are short-term biomarkers (residence time \( \leq 2 \) days), which should be characterized by larger variance ratio than biomarkers with longer residence times (Lin et al., 2005).

Moreover, previous studies reported much higher values of \( \lambda \) for biomarkers of styrene exposure. The retrospective investigation of MA and PGA concentrations in 331 workers from 8 factories manufacturing reinforced plastics in the Emilia Romagna region (Northern of Italy) between 1985 and 1999 showed intraclass correlations (ICCs) ranging from 0.00 to 0.74, with typical value around 0.5 (Symanski et al., 2001), which are equivalent to a range of \( \lambda \) between \(+\infty\) and 0.35, with a typical value of 1. A similar ICC range (from 0.00 to 0.752, corresponding to \( \lambda \) between \(+\infty\) and 0.33)
has been reported in a recent evaluation of six plastic manufacturing industries, where StyA, MA, PGA and their sum, blood styrene, and alveolar styrene were investigated during a time frame ranging from 1 week to 2 years (Symanski et al., 2007). Finally, the comparative evaluation of airborne styrene and MA in 12 fiberglass reinforced plastic workers, sampled three times at intervals of 1–4 months, resulted in a \( \lambda \) value of 0.434 and 0.908 (Liljelind et al., 2003).

The smaller values of \( \lambda \) in our investigation may reflect some differences in our study design. First of all, the sampling time of the present study was limited to 6 consecutive weeks vs. up to two decades in the retrospective study by Symanski et al. (2001). Second, a relatively large number of repeated measurements were available for our workers as compared to only two repeats reported by Symanski et al. (2007). And, finally, our workers performed the same job tasks during the relatively short period investigated whereas workers rotated through different tasks in the other studies (Symanski et al., 2001; Liljelind et al., 2003). Furthermore, the good precision and high sensitivity of analytical assays conducted in our laboratory may have reduced random errors that could have increased within-subject variability in the other studies.

Considering styrene biomarkers from a more general perspective, we found that, in addition to other advantages of MA and MA + PGA noted in the literature (e.g., abundance, good correlation with environmental exposure, high specificity and sensitivity, low background levels, simplicity of the assay), the low variance ratios of MA (\( \lambda = 0.178 \) for varnish workers and 0.210 for plastic workers) and MA + PGA (\( \lambda = 0.188 \) for both groups) observed in our study support their use as the most useful biomarkers of styrene exposure. Interestingly, M1 + M2 was also found to have a very small variance ratio (\( \lambda = 0.089–0.178 \)). Since urinary excretion of mercapturic acids M1 + M2 is modulated by the GSTM1 polymorphism, with subjects bearing the GSTM1pos genotype excreting 5–7-fold higher concentrations of M1 + M2 than subjects lacking GSTM1 activity (De Palma et al., 2001; Haufrad et al., 2001; Fustinoni et al., 2008), it is reasonable to expect that functional polymorphisms would increase between-subject variability, as we observed for M1 + M2 in our samples (\( \sigma^2_{y\nu} \) values were 2.90 and 1.32 for varnish and plastic workers, respectively, and the corresponding \( \lambda \) values were 0.178 and 0.165). This supports the conclusion of Lin et al. (2005) that the effect of functional polymorphisms leads to an increase in \( \sigma^2_{y\nu} \) of relevant biomarkers while \( \sigma^2_{y} \) is unaffected, with a resulting decreased in the variance ratio of the biomarker. On the other hand, the practical use of M1 + M2 is limited by the analytical challenge of measuring low concentrations of four mercapturic acids and the characterization of GSTM1 polymorphism is needed for correct interpretation of M1 + M2 levels.

The biomarkers characterized by the largest variance ratios were StyU and PHG in varnish workers (\( \lambda = 1.22 \) and 1.38, respectively) but not in plastic workers (\( \lambda = 0.197 \) and 0.535, respectively). This contrasting behavior in these groups of workers points to possible problems during sampling, storing and/or analysis of the samples.

Finally, we note that the variance ratio of StyA (\( \lambda = 0.147 \) and 0.271 in varnish and plastic workers, respectively) was in the same range as those of the best urinary biomarkers in our study. Thus, biomonitoring would appear to offer no obvious advantages over environmental monitoring for styrene in epidemiologic studies, and selection of air or biological monitoring should be based upon ease of use and other practical considerations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.
Acknowledgements

The present work was partially founded by the Italian Ministry of Health (PMS/19/01/U05) and by the Italian Institute for Safety and Health at Work (ISPESL, Research B58/97). We are indebted to Matteo Goldoni for statistical advice, and to the subjects who volunteered in the study.

References

American Conference of Governmental Industrial Hygienists (ACGIH); TLVs and BEIs based on the documentation of the threshold limit values for chemical substances and physical agents & biological indices. Cincinnati, USA: ACGIH, 2007.


