A case study of benzene urinary biomarkers quantification: the comparison between pre- and post-shift samples improves the interpretation of individual biological monitoring data

Nadia Miraglia[1]
Giuliana Genovese[1]
Pascale Basilicata[2]
Maria Pieri[1,2]
Angela Simonelli[1]
Rossella Guadagni[1]
Monica Lamberti[1]
Paola Pedata[1]
Antonio Acampora[2]
Nicola Sannolo[1]

[1]Department of Experimental Medicine - Section of Hygiene, Occupational Medicine and Forensic Medicine - Occupational Medicine Area - Second University of Naples, Italy
[2]Department of Advanced Biomedical Sciences - Section of Forensic Medicine, Histology and Anatomy, University of Naples "Federico II", Italy

Corresponding Author:
Giuliana Genovese
Department of Experimental Medicine - Section of Hygiene, Occupational Medicine and Forensic Medicine - Occupational Medicine Area - Second University of Naples
Via L. De Crecchio 7
80138 Naples, Italy
E-mail: dottssagenovese@gmail.com

Abstract

Introduction: **benzene is an ubiquitous pollutant of working and living environments. Hence occupational prevention strategies need to discriminate between different exposure sources. The aim of the study is to take into account confounding factors in the evaluation of occupational exposure to benzene by biological monitoring (BM) investigations in small working realities.**
Materials and Methods: **the BM of 14 workers of a refueling station was performed. Urinary benzene (UB) and trans, trans-muconic acid (*t,t*-MA) were used as biological markers. UB and *t,t*-MA determinations were performed by head space-solid phase microextraction followed by gas chromatography/mass spectrometry and High Performance Liquid Chromatography/UltraViolet detection.**
Urine collection was performed after work-shift (first BM campaign) as suggested by ACGIH. Subsequently, the urine collection was repeated before and after the work-shift (second BM campaign).
Results: **during the first BM campaign, contrasting results were found. Some workers showed low UB concentrations with respect to *t,t*-MA ones and *vice versa*, and although most subjects presented UB and *t,t*-MA levels below Biological Equivalents values and ACGIH's BEI, various exceptions were found. Biomarkers concentration decrements were observed by comparing pre- and post-shift samples (second BM campaign).**
Discussion: **according to the ACGIH, the high biomarkers levels found in post-shift urine would suggest occupational exposure to benzene. Nevertheless, results obtained by monitoring post-shift urine only, without knowing individual background levels do not facilitate data interpretation.**
Findings from the second BM campaign suggest that biological monitoring strategies aimed to evaluate the occupational exposure of individuals to ubiquitous hazardous chemicals, need the measurement of biomarkers concentration both before and after the work-shift.

KEY WORDS: benzene, biological monitoring, biomarkers, urine specimen collection.

Introduction

Data from epidemiological studies evidence benzene toxicity to humans. In fact, it is associated with the development of acute non-lymphocytic leukemia (1, 2), multiple synchronous primary malignancies (3), chromosomal aberrations (4) and a progressive degeneration of the bone marrow (5).
Benzene is a common industrial chemical and a component of tobacco smoke and of gasoline. In fact, it is widely used as chemical intermediate (for instance, in the production of monomers for polymer industry) and is a constituent of crude oil and fuels (<1% v/v in the United States and European Union). Moreover, benzene is distributed in the environment by vehicles engine emissions. Smoking tobacco is regarded as another major source of environmental benzene exposure. In particular, mainstream and sidestream cigarette smoke (smoke emitted by slow combustion of cigarettes irrespective of aspiration by the smoker) has been calculated to emit 6-73 and 120-480μg benzene/cigarette into ambient air, respectively (6). Hence, benzene has been becoming an ubiquitous pollutant of the outdoor and indoor human environ-

ment, and benzene occupational or environmental exposure concerns a large population (7-11).
Therefore health risks prevention strategies allowing to discriminate between occupational and non-occupational exposure are essential.
The American Conference of Governmental Industrial Hygienists (ACGIH) classifies benzene in group A1 (carcinogen to human) and for prevention of health risks arising from occupational exposure to benzene, defines a threshold limit value-time weighted average (TLV-TWA) of 0.5ppm (12).
ACGIH has also adopted post-shift urinary *trans, trans*-muconic acid (*t,t*-MA) or S-phenylmercapturic acid (S-PMA) as benzene exposure biomarkers, suggesting concentrations levels of 500 and 25μg/g creatinine as Biological Exposure Index (BEI), respectively (12).
Besides exposure biomarkers proposed by ACGIH, unmetabolised benzene (measured in blood, breath and urine) has been widely used as sensitive and specific marker for the biological monitoring of benzene exposed workers (13-16), and, in particular, urinary benzene (UB) is currently used in a lot of biological monitoring investigations (17-24). When ACGIH does not suggest BEI, it is possible to evaluate occupational exposure by comparing the biomonitoring data with Biomonitoring Equivalents (BEs), i.e. "the concentration of chemical (or metabolite) in blood, urine or some other tissue, consistent with exposure guidance values such as a Tolerable Daily Intake (TDI), Reference Dose (RfD), Reference Concentration (RfC), or risk specific doses (cancer)" (25). BE values for benzene in urine range from 50 to 1420ng/L, depending upon the underlying non-cancer risk assessment used in deriving the BE itself (26).
As for the other biomarkers (18, 23), urinary benzene was found to be highly affected by cigarette smoking at the observed low environmental benzene concentrations. In fact, many Authors showed that levels of urinary benzene are always significantly higher in smokers than in non-smokers and increase with the number of smoked cigarettes (14, 18, 20, 24).
Within this context, preliminary survey was performed by comparing urinary biomarkers levels in pre- and post-shift samples in biological monitoring (BM) investigations. The objective of the survey was to find how biomarkers levels data, related to small working realities (i.e. with few exposed workers) can be interpreted taking into account the influence of confounding factors, such as smoking habits in particular. Findings from the case-study here reported were aimed to facilitate individual data interpretation in occupational exposure evaluation.

Materials and methods

Chemicals and instrumentation

Benzene was purchased from Merck (Darmstadt, Germany), *t,t*-MA and benzoic acid (used as internal standard) from Sigma-Aldrich (Milwaukee, WI, USA). Esadeuterated benzene and HPLC grade solvents (ethylacetate, hydrochloric, picric and formic acids) as well as 10ml vials and silicone/Teflon lined (0.1mm thickness) septa "superior standard" were from Carlo Erba (Milan, Italy). SPME fibers (fused-silica fibers 10mm long, coated with an 85μm film thick layer of polydimethylsiloxane/carboxen) and the fiber holder were from Supelco (Bellafonte, PA, USA).
GC/MS-SIM analyses were carried out by using a gas chromatograph GC 8000 Series, interfaced with a single quadrupole mass detector Voyager, equipped with an Electron Ionization source (Fisons Instruments, Milan, Italy). The gas chromatograph was equipped with a split/splitless injector (0.75mm i.d., inlet liner for SPME) from Supelco (Bellafonte, PA, USA) and a CP-PoraBOND Q (25m length x 0.25mm i.d. x 3µm film thickness) capillary column from Varian (Palo Alto, CA, USA).
HPLC/UV analyses were carried out with a HP 1100 HPLC System, equipped with a variable wavelength detector, produced by Agilent Technologies (Palo Alto, CA, USA). An ODS Hypersil C18 column, 250mm length x 3mm i.d., 3μm particle size, (Thermo Electron Corporation, Rodano, Milan, Italy) was used for the quantitative determination of *t,t*-MA.
Creatinine was quantified by using the Cary 60 UV-Vis Spectrophotometer (Agilent Tecnologies, Palo Alto, CA, USA), operating from 190nm to 1100nm.

Investigated workers and samples collection

BM investigations were planned in agreement with the occupational physician of the examined refueling station, within the health surveillance program. Fourteen workers were asked to give detailed information about eating and smoking habits (cigarettes smoked during the day), specific tasks (tanks refuelling, fuels supply, office work), and personal protective equipments. No inclusion/exclusion criteria were used, and smoker workers were asked to abstain to smoke cigarettes during the monitoring.
Then, extemporaneous urine specimens were collected in polypropylene containers. In the first BM campaign, urine collection was performed only at the end of work-shift, subsequently before and after the work-shift (second BM campaign). Each sample was divided into five aliquots. Three aliquots were used for benzene quantification, the other two for the determination of *t,t*-MA and creatinine, respectively.

Urinary benzene and t,t-MA analyses

For the determination of urinary benzene by HS-SPME/GC/MS, the Basilicata et al. method was used (17).
For the determination of *t,t*-MA by HPLC/UV, one urine aliquot (10mL) of each investigated worker was transferred in a polypropylene tube and mixed with 2-3 drops of HCl 37% as a preserving agent. Subsequently, 1mL of each urine sample was added with a methanolic solution of benzoic acid, as internal stan-

dard, so that a constant urinary concentration of 50ng/μL was obtained. The determination of urinary *t,t*-MA was carried out by pre-purification of urine with liquid-liquid extraction. In detail, 1mL of urine samples was mixed with 4mL of ethylacetate and then centrifuged (4000rpm for 15min), the procedure was repeated twice. The supernatant was recovered and dried under a nitrogen stream. Then samples were frozen at -20°C and analyzed within a week. The HPLC/UV analysis was performed by dissolving each dry sample with 250μL of 0.05M formic acid, then an aliquot of 50μL was loaded into the HPLC/UV system, setting a flow of 0.4mL/min and a wavelength of 264nm. An aqueous solution of formic acid 0.05M and methanol were used as solvent A and B, respectively. The elution program was: isocratic elution at 10% B for 5 min, linear gradient from 10 to 100% B over 15 min.

Urinary creatinine spectrophotometric analysis

Urine aliquots used for the determination of urinary creatinine were frozen (-20°C), without preserving agents, until analysis. The values of creatinine concentrations were used to assure the validity of the samples, according to the acceptable range (0.3g/L ≤ creatinine ≤ 3.0g/L) indicated by the World Health Organization (27). Besides, creatinine was quantified in order to normalize the *t,t*-MA concentrations with respect to creatinine levels, as suggested by ACGIH. In contrast, benzene amounts were not normalized because the benzene excretion occurs by diffusion and is not influenced by renal function, in fact BEs values are expressed in ng/L (26).
The determination of urinary creatinine was performed by using Jaffe's colorimetric method (28). Briefly, each urine sample was diluted 50 times with deionized water. Directly into the cuvette, 100μL of diluted sample were mixed with 1mL of 25mM picric acid/0.4M sodium anhydrous hydroxide (50/50, v/v). The average increase of absorbance at wavelength 500nm in the range 30-90s after the start of the reaction was recorded.

Results

Post-shift urine collection

According to ACGIH, the first BM campaign was carried out by collecting only post-shift urine, and benzene and *t,t*-MA concentrations were measured in urine from the 14 investigated subjects exposed to benzene. In order to understand if the benzene concentrations found in urine could be related to hypothetical exposure levels, a theoretic scale about the expected exposure levels was established: very low (administrative employees, workers 1 and 2), low (manager, worker 3), medium (cleaner and pumps attendant for half-shift, worker 4), high (pumps attendants, workers 5-14).
Results from the BM, as well as theoretic exposure levels and smoking habits are reported in Table 1, no personal protective equipment was used.

Pre- and post-shift urine collection

Biological monitoring investigations were repeated by collecting urine samples before and after work-shift. In this case, an environmental monitoring was also carried out in order to put in evidence the occupational exposure with respect to benzene absorption coming from smoking habits and lifestyle (data not shown). No different exposure levels were observed by analyzing environmental data, nevertheless this was not sufficient to assess the absence of occupational exposure for each individual. In addition, actually the Italian Leg-

Table 1 - Smoking habits, creatinine levels and benzene and *t,t*-MA concentrations in post-shift urine from benzene occupationally exposed workers.

Worker	Cigarette/day	[creatinine] g/L	[*t,t*-MA] μg/g creatinine	[benzene] ng/L
1 I [a]	0	0.8	33	82
2 I [a]	0	0.6	299	249
3 I [b]	10	1.1	211	321
4 I [c]	20	3.0	70	**1960**
5 I [d]	0	2.9	105	176
6 I [d]	15-20	0.3	**1022**	109
7 I [d]	3-4	0.9	390	295
8 I [d]	10-20	2.3	63	**1800**
9 I [d]	10	0.5	**503**	113
10 I [d]	20	2.9	114	288
11 I [d]	0	0.5	334	214
12 I [d]	20	1.6	212	1130
13 I [d]	10	2.9	111	746
14 I [d]	10-20	1.8	44	898

Theoretic benzene exposure levels based on worker specific tasks: [a] Very low; [b] Low; [c] Medium; [d] High.
I: first biological monitoring campaign.

islation considers biological (not environmental) monitoring examinations "when biomarkers have been established for a specific hazardous agent" (Legislative Decree 81, 2008) (29). Results coming from BM are shown in Table 2.

Discussion

Various research studies are reported in Literature regarding the evaluation of the occupational exposure to benzene by the measurement of biomarkers levels in post- shift samples only (19, 20, 22, 24). These studies are based on a sample size of at least 24-45 subjects, hence results can be statistically interpreted, even if investigated subjects are further divided in smokers and non-smokers. The here reported findings agree with literature data as far as regards the smoking habits influence (higher urinary biomarkers levels correspond to smokers), nevertheless show that in the case of small working realities (no literature data available), BM individual data may not be easy to interpret.

In fact, during the first BM, contrasting results were found. For instance, worker 1 showed remarkably lower biomarkers levels with respect to worker 2, even if both were non-smokers and had the same theoretic exposure level. As well as worker 10 presented lower biomarkers levels with respect to worker 12, and both smoked 20 cigarettes a day and had the same theoretic exposure level. As a consequence, no hypothetical relations between biomarkers concentrations and either theoretic exposure levels or smoking habits could be found.

In contrast, some workers showed low UB concentrations with respect to *t,t*-MA ones and *vice versa*, and actually no relations between the biomarkers excreted amounts should have been found, as expected owing to their different excretion kinetics.

Table 2 - Smoking habits, creatinine levels and benzene and *t,t*-MA concentrations in pre- and post-shift urine from benzene occupationally exposed workers.

Worker	Cigarette/day	Time of collection	[creatinine] g/L	[*t,t*-MA] *μ*g/g creatinine	[benzene] ng/L
1 II [a]	0	before shift	0.9	38	85
		after shift	0.8	41	79
2 II [a]	0	before shift	0.5	300	290
		after shift	0.7	310	281
3 II [b]	10	before shift	0.9	200	210
		after shift	1.2	232	308
4 II [c]	20	before shift	2.8	145	183
		after shift	1.2	383	103
5 II [d]	0	before shift	3.0	193	741
		after shift	2.0	140	357
6 II [d]	15-20	before shift	0.6	**1038**	217
		after shift	1.8	**766**	489
7 II [d]	3-4	before shift	1.5	41	791
		after shift	2.7	36	343
8 II [d]	10-20	before shift	0.8	115	399
		after shift	3.0	209	159
9 II [d]	10	before shift	2.7	230	343
		after shift	2.5	248	570
10 II [d]	20	before shift	2.3	79	65
		after shift	1.7	97	500
11 II [d]	0	before shift	1.3	77	254
		after shift	1.9	99	82
12 II [d]	20	before shift	2.4	99	**1920**
		after shift	2.0	60	823
13 II [d]	10	before shift	0.9	270	470
		after shift	1.1	158	495
14 II [d]	10-20	before shift	1.8	116	**1782**
		after shift	1.6	80	1415

Theoretic benzene exposure levels based on worker specific tasks: [a] Very low; [b] Low; [c] Medium; [d] High.
II: second biological monitoring campaign.

Most investigated subjects presented UB and t,t-MA levels below BEs values and ACGIH's BEI, respectively, suggesting a non-occupational exposure. Nevertheless, four exceptions were found, either with exceeding UB (workers 4 and 8) or with exceeding *t,t*-MA (workers 6 and 9) levels. Various hypotheses could explain these results. For instance, high *t,t*-MA concentrations may depend on the urinary creatinine levels close to the lower limit of the acceptable range of urine samples, as well as they could be affected by specific preservatives in food. Moreover, all workers with high urinary levels of benzene or *t,t*-MA were smokers (from 10 to 20 cigarettes/day), hence high biomarkers concentrations cannot be exclusively attributed to occupational exposure to benzene. Actually, results obtained by monitoring post-shift urine only without knowing individual background levels of urinary benzene and *t,t*-MA for each examined worker, do not facilitate the interpretation of data and do not help the occupational physicians to discriminate between occupational and non-occupational exposure.

In the second BM campaign, as well as for the first one, biomarkers urinary excretion levels could not be explained on the basis of expected exposure levels or smoking habits, nevertheless, a better data interpretation could be given from the comparison of pre- and post-shift measurements.

Following occupational exposure, an increment in biomarkers concentrations in post-shift urine with respect to pre-shift ones would have always been expected. In contrast, most workers (both sundry smokers and non-smokers) showed a remarkable decrement of at least one of the two investigated biomarkers, suggesting a non-occupational exposure. In particular, results from non-smoking workers 5 and 11 are completely independent from smoking habits, as a consequence, the observed decrement of biomarkers levels strongly suggests an exposure to benzene due to other factors (such as food, environmental pollution, passive smoking, etc.) rather than to an occupational one.

Also in the second BM campaign, some results were higher than ACGIH's BEI and BEs for t,t-MA and UB, respectively, but in this case data can be interpreted as follows.

Workers 12 and 14 had high UB concentrations (beyond BEs) in pre-shift urine, but these values decreased during the working day, as evident from UB levels of post-shift urine, demonstrating that there is no occupational exposure. Nevertheless, in the cases of workers 12 and 14, if only post-shift urine had been monitored, results would have led to the same conclusion because post-shift urine UB levels are below BEs.

In contrast, the importance of measuring both pre- and post-shift urine is illustrated by workers 6. In fact, he presented a concentration of t,t-MA in post-shift urine higher than the BEI proposed by ACGIH, and an occupational exposure could be suspected. Nevertheless, a higher concentration value was found in pre-shift urine, demonstrating that benzene absorption must be attributable to factors other than the occupational one. Besides, worker 6 showed a decrease of t,t-MA and an increase of UB (and vice versa in other workers), confirming, as previously hinted, that no relation between the increase and the decrease of biomarkers urinary concentration could be highlighted when small working contexts (individual data) are analyzed, depending on longer urinary half-life of t,t-MA (4-6 hr) when compared with UB one (2-3 hr).

The decrease of UB during the work-shift surely suggests a non-occupational exposure, but it may also be linked to other factors, such as the circadian rhythms in the biochemical activity of organs and tissues (30). Due to the nocturnal drop in production of urine and metabolites, the concentration of circulating benzene (released from storage tissues) increases. Later, the resumption of kidney and liver activities subtracts benzene from circulation. Therefore, in morning samples, the concentration of UB should reflect the amount present in blood, while during the day, benzene concentration decreases. Nevertheless, even if the circadian rhythms had influenced UB levels, in case of occupational exposure to benzene, the observed UB decrements would have been less remarkable.

In conclusion, when human healthcare deals with ubiquitous chemical agents affected by confounding factors, prevention strategies aimed to exposure levels reduction should be based, first of all, on the individuation of exposure sources. The here reported case-study is an example of this consideration, applied in a small occupational exposure context, where no statistical analyses can be performed. Actually, the limit of here reported findings consists in the non generalizability of obtained data, requiring further studies on large scale. Nevertheless, results show that, in the case of benzene (an environmental pollutant also present in cigarette smoking), the discrimination between occupational and non occupational exposure could be facilitated by the comparison between exposure biomarkers levels found in pre- and post- shift urine, and biological monitoring investigations should consider individual background levels of exposure biomarkers.

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