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Applicability of urinary benzene to biological monitoring of occupational and environmental exposure to very low benzene concentrations

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ABSTRACT.* Objective.** ***To verify whether urinary benzene is an applicable biomarker of occupational exposure to very low concentrations of benzene, considering the influence of cigarette smoke and benzene-toluene co-exposure.
Materials and methods. ***23 filling station attendants with occupational exposure to benzene and 31 controls were analyzed. Occupational and environmental exposure was monitored and t,t-muconic acid (t,t-MA), S-phenylmercapturic acid (SPMA), urinary benzene and creatinine in the urine samples were tested.***
Results. ***Occupational exposure to benzene and toluene was significantly higher in the filling station attendants than in the controls, whereas t,t-MA, SPMA and urinary benzene were not different in the two groups. Instead, the smoker group showed significantly higher values for the above biomarkers than the non-smoker group, each of which included both exposed workers and controls. SPMA was dependent on airborne benzene and cigarette smoking, and urinary benzene only on cigarette smoking, while t,t-MA was not dependent on either of these variables.***
Conclusions. ***At very low concentrations of occupational exposure to benzene, urinary benzene is less valid than SPMA as a biomarker, even if both are strongly influenced by smoking habit. Abstention from smoking should therefore be recommended for at least two hours before urine collection.***

Key words: *Biological monitoring, Urinary Benzene, S-phenylmercapturic acid (SPMA), t,t-muconic acid (t,t-MA).*

RIASSUNTO. Applicabilità del benzene urinario nel monitoraggio biologico dell'esposizione occupazionale ed ambientale a concentrazioni molto basse di benzene.
Obiettivo. **Verificare l'applicabilità del benzene urinario quale biomarcatore di esposizione occupazionale a concentrazioni molto basse di benzene, considerando l'influenza che hanno su di esso il fumo di sigaretta e la co-esposizione benzene-toluene.**
Materiali e metodi. **Sono stati analizzati 23 benzinai occupazionalmente esposti a benzene e 31 controlli non esposti occupazionalmente al tossico. L'esposizione occupazionale e ambientale a benzene è stata misurata mediante campionatori personali passivi e l'acido *t,t*-muconico (*t,t*-MA), l'acido S-fenilmercapturico (SPMA), il benzene urinario e la creatinina sono stati determinati nei campioni di urine raccolti da tutti i soggetti esaminati.**
Risultati. **L'esposizione occupazionale a benzene e a toluene è risultata significativamente più elevata nei benzinai rispetto ai controlli, laddove *t,t*-MA, SPMA e benzene urinario non**

Introduction

Despite the progressive reduction in the concentrations of benzene achieved in work and living environments in recent years, this aromatic hydrocarbon is still an occupational and airborne pollutant that arouses extreme toxicological concern, owing to its proven carcinogenic properties (1).

In western countries, occupational exposure to benzene can now be defined as low or very low, being about 2 or 3 orders of magnitude lower than the TLV-TWA proposed by the American Conference of Governmental Industrial Hygienists (ACGIH) (2). In the environment, benzene is present in automobile exhaust fumes and cigarette smoke, both important sources of pollution of the outdoor and indoor environments, respectively (3). Moreover, benzene is nearly always present in co-exposure with toluene, both in work and living environments.

In view of the influence of non occupational sources of benzene like cigarette smoking or confounding factors such as dietary habits and benzene-toluene co-exposure on the benzene biomarkers for occupational exposure to the above-described airborne benzene concentrations, doubts have arisen as to the validity of using the urinary concentrations of *t,t*-muconic acid (*t,t*-MA) and S-phenylmercapturic acid (SPMA) as biomarkers of the internal dose of this toxicant (2, 4, 5).

Therefore, to monitor occupational exposure to low or very low concentrations of benzene, urinary benzene has been proposed as a new biological index. However, further study is needed before it can be reliably used as a biomarker of the internal dose, as observed by other authors (6, 7), and as demonstrated also in our recent study of workers with ranges of exposure to airborne benzene of between 4.5 and 1017 μg/m^3. At the observed low airborne concentrations, urinary benzene showed a largely comparable validity to SPMA for monitoring occupational exposure to benzene, although both biomarkers were strongly influenced by cigarette smoke (8).

To assess the applicability of urinary benzene as a biomarker of occupational exposure to lower concentrations

hanno mostrato differenze nei due gruppi. Viceversa, i suddetti indicatori biologici hanno mostrato concentrazioni urinarie più alte nei fumatori rispetto ai non fumatori, includendo entrambi i gruppi esposti e non esposti. È risultata una dipendenza dell'SPMA dal benzene ambientale e dal fumo di sigaretta e del benzene urinario soltanto dal fumo di sigaretta, mentre il *t,t*-MA non è risultato dipendente da alcuna di queste due variabili.
Conclusioni. **Alle concentrazioni molto basse di esposizione occupazionale a benzene osservate, il benzene urinario risulta essere un biomarcatore meno valido rispetto all'SPMA, anche se entrambi sono fortemente influenzati dal fumo di sigaretta. L'astensione dal fumo di sigaretta, pertanto, dovrebbe essere raccomandata almeno nelle due ore precedenti la raccolta delle urine.**

Parole chiave: Monitoraggio biologico, Benzene urinario, Acido S-fenilmercapturico (SPMA), Acido *t,t*-muconico (*t,t*-MA).

than those reported in the previous work, filling station attendants exposed to airborne benzene concentrations ranging between 4.5 and 66.3 µg/m^3 and controls were examined. Urinary benzene was compared with urinary *t,t*-MA and SPMA, and the influence of cigarette smoke and co-exposure to benzene and toluene was also assessed.

Subjects and methods

Subjects

The study sample consisted of 23 male filling station attendants occupationally exposed to benzene and 31 male workers with no occupational exposure (controls). All participants completed a questionnaire probing personal data, current and previous job descriptions, lifestyle habits, possible exposure to non-occupational sources of benzene and toluene. Informed written consent to take part in the study was obtained from all subjects before enrollment.

Exposure assessment and analytical procedures

Air sampling

Exposure to airborne benzene and toluene was assessed in all subjects by passive personal sampling, using radial diffusion samplers (Radiello®) containing an active carbon cartridge, that were worn for eight hours. Analysis of the Radiello® vials was performed by Gas Chromatography, with a Flame Ion Detector (GC-FID), applying a modified NIOSH method (9). The detection limit of the procedure for both benzene and toluene was 3 µg/m^3. All analyses were conducted blinded.

Urine sampling

On the same day as the air sampling, urine samples were obtained at the end of the work shift from the exposed groups and the controls for measurement of the biological indexes of exposure to benzene (*t,t*-MA, SPMA, urinary benzene). All the analyses were conducted blinded. The analysis of *t,t*-MA was carried out with the HPLC-UV method after solid phase extraction (10). The detection limit of the procedure was 10 µg/L. The analytical determination of urinary SPMA was performed by liquid chromatography/electrospray tandem mass spectrometry (HPLC-ESI-MS/MS), as reported by Sabatini *et al*. (11). The limit of detection was 0.20 µg/L. The determination of urinary benzene was done by Gas-Chromatography/Mass Spectrometry (GC/MS) (12). The limit of detection was 0.02 µg/L. Analyses of urinary creatinine, based on the Benedict/Behre test, were performed using a DCA 2000®+analyzer (13).

Statistical analyses

Statistical analyses were done using the SPSS program (version 14.0, Chicago, IL, USA). Values below the detection limit were reported in the database at a value corresponding to one-half of the detection limit. The Kolmogorov-Smirnov test was used to check the normal distribution of all variables. Non normally distributed variables were analyzed by parametric tests after logarithmic transformation or by non parametric tests. Spearman's test was used for correlation analysis. Any dependency of the various biological markers on independent variables was assessed by multiple linear regression models. The level of significance was set at $p<0.05$.

Results

No significant differences were observed between the filling station attendants and the controls in terms of age, body mass index (BMI), alcohol consumption and number of cigarettes smoked per day (table I).

Airborne benzene and toluene concentrations were significantly higher in the filling station attendants than in the controls (table II). Instead, there were no differences in the concentrations of *t,t*-MA, SPMA and urinary benzene between the exposed subjects and controls.

After subdividing all the examined subjects into smokers and non smokers, study of the relations between cigarette smoking and both the levels of airborne benzene and the urinary concentrations of its biological indicators did not reveal significant differences as regards exposure to airborne benzene, but smokers had significantly higher concentrations of *t,t*-MA, SPMA and urinary benzene than non smokers (table III). Moreover, after stratifying exposed subjects and controls by cigarette smoking, this confounder was found to be associated, by two-way analysis of variance, with both SPMA and urinary benzene levels (model and cigarette smoke, both $p<0.002$), unlike occupational exposure to benzene, whereas no association with *t,t*-MA was demonstrated (model $p=0.927$).

Airborne benzene and toluene were well correlated, even if this finding was significant only when examining the whole sample together, and exposed subjects (for both groups, rho=0.94; $p<0.001$). The number of cigarettes smoked per day and of those smoked during the work shift were strongly correlated with *t,t*-MA, SPMA and urinary benzene in all 3 groups, with the exception of *t,t*-MA and the number of cigarettes smoked per day by controls,

Table I. ***Characteristics of the two subject groups examined***

	FILLING STATION ATTENDANTS					CONTROLS				
	N.	Mean±SD	GM	Median	Range	N.	Mean±SD	GM	Median	Range
Age (years)	23	40.7±9.9	39.5	40.0	19-60	31	41.7±9.1	40.7	43.0	22-62
Body Mass Index (BMI) (Kg/m^2)	23	26.0±3.9	25.7	26.5	20.3-37.2	31	26.7±4.8	26.3	25.8	20.9-44.4
Alcohol										
Teetotal	7	30.4%				10	32.3%			
<10 g/day	7	30.4%				9	29.0%			
>10 g/day	9	39.2%				12	38.7%			
N. cigarettes/day	11	18.8±10.9	14.8	20.0	2-40	16	16.6±10.6	13.9	11.0	5-40
N. cigarettes/sampled shift	11	13.1±10.2	–	10.0	0-40	16	7.6±5.4	–	6.0	0-20

GM = Geometric mean

Table II. ***Airborne concentrations of benzene and toluene and the different urinary biomarkers of exposure to benzene in the two groups***

	FILLING STATION ATTENDANTS					CONTROLS				
	N.	Mean±SD	GM	Median	Range	N.	Mean±SD	GM	Median	Range
Airborne benzene (µg/m^3)[a]	23	23.5±17.4	17.6	20.9	4.5-66.3	31	4.6±2.6	3.9	4.3	<3.0-11.5
Airborne toluene (µg/m^3)[a]	22	144.3±108.6	108.7	130.9	19.0-479.0	9	15.2±13.0	11.6	13.1	<3.0-47.4
Urinary *t,t*-muconic acid (µg g creat^{-1})	23	86±34	77	89	11-157	31	93±132	61	59	13-734
Urinary SPMA (µg g creat^{-1})	23	0.79±0.77	0.47	0.56	0.05-3.33	31	0.65±1.00	0.27	0.22	0.03-4.48
Urinary benzene (µg L^{-1})	23	0.62±0.74	0.27	0.21	0.04-2.87	31	1.23±2.63	0.21	0.12	<0.02-11.40

GM = Geometric mean; [a]p<0.001

Table III. ***Airborne concentrations of benzene and its urinary biomarkers of internal dose in all the subjects, subdivided by smoking habit***

	SMOKERS					NON SMOKERS				
	N	Mean±SD	GM	Median	Range	N	Mean±SD	GM	Median	Range
Airborne benzene (µg/m^3)	27	11.8±11.1	7.8	8.0	<3.0-41.9	27	13.6±17.9	7.0	4.9	<3.0-66.3
Urinary *t,t*-muconic acid (µg g creat^{-1})[a]	27	100±60	79	90	13-310	27	80±133	57	57	11-734
Urinary SPMA (µg g creat^{-1})[b]	27	1.09±0.98	0.71	0.80	0.03-4.48	27	0.32±0.63	0.16	0.14	0.04-3.33
Urinary benzene (µg L^{-1})[b]	27	1.85±2.65	0.87	1.01	0.04-11.40	27	0.09±0-08	0.07	0.06	<0.02-0.37

GM = Geometric mean; [a]p<0.05; [b]p<0.001

which did not quite reach the level of significance (table IV). Airborne benzene was correlated with *t,t*-MA, SPMA and urinary benzene in the whole sample examined together, and with SPMA also in the controls. The three urinary biomarkers of exposure to benzene were all correlated in the whole sample, even if a correlation in all three groups was present only between SPMA and urinary benzene. The time between the last cigarette smoked and urine collection was negatively correlated with SPMA and urinary benzene in the whole sample examined together, whereas alcohol consumption was not correlated with *t,t*-MA, SPMA or urinary benzene in any of the groups.

The dependency of *t,t*-MA, SPMA and urinary benzene on the independent variables age, BMI, alcohol intake, number of cigarettes/day and airborne benzene was studied by applying different multiple regression models to the whole sample (table V). The results showed a dependency of the levels of urinary benzene only on cigarette smoking and of SPMA on cigarette smoking and airborne benzene, whereas *t,t*-MA was not dependent on any of the variables considered.

The simultaneous influence of cigarette smoking and co-exposure to different levels of toluene on the relationship between urinary benzene and *t,t*-MA and SPMA, respectively, was assessed by subdividing all examined subjects by smoking habit and exposure to airborne levels of toluene below or above 65.5 µg/m^3, that was the median value of the latter. Two-way analysis of variance showed that the urinary benzene / *t,t*-MA ratio is associated exclusively with cigarette smoking, whereas the urinary benzene / SPMA ratio is not associated with either of the above variables (table VI).

Table IV. ***Spearman's correlation between smoking, airborne benzene and its biomarkers of internal dose in the entire sample examined together, and subdivided into filling station attendants and controls***

		N. cigarettes /day	N. cigarettes /sampled shift	Time between last cigarette and urine collection	Alcohol	Airborne benzene	Urinary *t,t*-muconic acid	Urinary SPMA
N. cigarettes /day	Total Attendants Controls	1	–	–	–	–	–	–
N. cigarettes /sampled shift	Total Attendants Controls	0.94[c] 0.95[c] 0.92[c]	1	–	–	–	–	–
Time between last cigarette and urine collection	Total Attendants Controls	-0.51[a] -0.40 -0.60[a]	-0.47[a] -0.01 -0.65[b]	1	–	–	–	–
Airborne benzene	Total Attendants Controls	0.15 0.40 -0.07	0.38[a] -0.06 0.11	-0.34 -0.74[b] -0.32	0.04 -013 0.18	1	–	–
Urinary *t,t*-muconic acid	Total Attendants Controls	0.40[b] 0.52[a] 0.35	0.43[c] 0.47[a] 0.38[a]	-0.15 0.11 -0.14	-0.05 -0.04 -0.06	0.31[a] -0.11 0.20	1	–
Urinary SPMA	Total Attendants Controls	0.65[c] 0.66[c] 0.69[c]	0.70[c] 0.66[c] 0.73[c]	-0.43[a] -0.46 -0.45	0.17 0.06 0.23	0.47[c] 0.22 0.58[c]	0.34[a] 0.15 0.37[a]	1
Urinary benzene	Total Attendants Controls	0.78[c] 0.80[c] 0.78[c]	0.78[c] 0.80[c] 0.78[c]	-0.45[a] -0.44 -0.44	0.04 -0.10 0.19	0.32[a] 0.23 0.33	0.33[a] 0.49[b] 0.15	0.68[c] 0.59[b] 0.68[c]

[a]p<0.05; [b]p<0.01; [c]p<0.001

Table V. ***Multiple linear regression applied to the entire study sample***

	t,t-muconic acid				SPMA				Urinary benzene			
	b	SE(b)	t	p	b	SE(b)	t	p	b	SE(b)	t	p
Age (years)	–	–	1.57	0.124	–	–	0.33	0.742	–	–	0.58	0.564
BMI (Kg/m^2)	–	–	1.09	0.365	–	–	0.93	0.359	–	–	0.59	0.561
N. cigarettes/day	–	–	2.46	0.017	0.064	0.013	4.91	<0.001	0.106	0.016	6.50	0.001
Airborne benzene (µg/m^3)	–	–	0.95	0.345	0.025	0.009	2.67	0.010	–	–	1.22	0.229
	F	p		R^2	F	p		R^2	F	p		R^2
Model	1.93	0.121		–	8.60	<0.001		0.47	10.90	<0.001		0.53

Table VI. ***Ratio between urinary benzene and SPMA and t,t-muconic acid, in the subjects subdivided by smoking habit and by levels of exposure to toluene above or below the median concentration (66.5 µg/m^3)***

	Airborne toluene	SMOKERS					NON SMOKERS						F	p
		N	Mean±SD	GM	Median	Range	N	Mean±SD	GM	Median	Range			
Urinary benzene /SPMA	Low exposure	6	1.55±1.05	1.23	1.49	0.17-3.15	10	1.04±1.19	0.49	0.73	0.01-4.00	Model	1.48	0.243
												Exposure	0.19	0.670
	High exposure	8	1.38±1.24	0.99	1.03	0.32-4.07	7	0.43±0.18	0.39	0.45	0.18-0.63	Smoking	4.41	0.045
												Interaction	0.02	0.894
	Total	14	1.45±1.24	1.05	1.12	0.17-4.07	17	0.79±0.79	0.45	0.57	0.01-4.00			
Urinary benzene /*t,t*-muconic acid	Low exposure	6	14.8±10.9	10.8	10.7	2.0-30.6	10	1.5±0.9	1.3	1.4	0.6-3.6	Model	19.80	<0.001
												Exposure	0.29	0.593
	High exposure	8	11.7±6.9	10.2	11.6	3.8-20.8	7	2.4±1.7	1.8	1.4	0.6-5.1	Smoking	55.60	<0.001
												Interaction	0.59	0.448
	Total	14	13.0±6.2	10.5	11.1	2.0-30.6	17	1.9±1.3	1.5	1.4	0.6-5.1			

Discussion

This research addressed the applicability of urinary benzene to biological monitoring of occupational exposure to very low concentrations of benzene. This biomarker was compared with the traditional biomarkers of internal dose *t,t*-MA and SPMA, and the influence of cigarette smoking and co-exposure to low concentrations of toluene was also assessed.

Very low benzene concentrations were observed in our filling station attendants, ranging from 4.5 to 66.3 µg/m^3, in line with other data in literature on exposure of these workers in western nations (14, 15). Nevertheless, the concentrations were still significantly higher in the filling station attendants than in the controls, even if there was a partial overlap between the two ranges. Environmental monitoring is therefore able to differentiate between exposed workers and controls, albeit only at group level, even in conditions of occupational exposure to very low concentrations of benzene.

Occupational exposure to toluene was also found to be very low, about 3 orders of magnitude lower than the limit proposed by the SCOEL and 2 orders lower than the one proposed by the ACGIH (2, 16).

At the airborne benzene concentrations observed in our filling station attendants, a prevalent role in conditioning the urinary excretion of *t,t*-MA was hypothesized for the diet, as compared to occupational benzene and cigarette smoking. This is attributable to eating foods containing sorbic acid (17). In fact, regression analysis did not show any dependency of *t,t*-MA both on the airborne levels of benzene and on cigarette smoking, probably due to the greater influence of the diet in conditions of a declining influence of exposure to airborne benzene.

Instead, at the same airborne benzene concentrations observed in the filling station attendants, SPMA resulted a valid biomarker, with a dependency on both airborne benzene and cigarette smoking (R^2=0.47), but not on the BMI. This suggests that the hypothesis that the fatty mass acts as a temporary deposit of benzene might have little importance at ever lower benzene concentrations.

Cigarette smoke is a main source of benzene exposure since in the "mainstream" smoke of a single cigarette the benzene content is equal to 28.0-105.9 µg and in the "sidestream" smoke it is equal to 70.7-134.3 µg, varying according to the brand (18). However, passive personal sampling using radial diffusion samplers (Radiello®), is not able to reveal the emission of benzene in the smoke (19, 20). For this reason, in smokers there is a discrepancy between the benzene exposure resulting from environmental monitoring, and the effective benzene quantity introduced, as indicated by the urinary concentrations of the different markers of the internal dose used in biological monitoring. This is particularly important in conditions of very low exposure to the toxicant like those we have observed, where the quantitative contribution of cigarette smoking as a source of benzene tended to be prevalent in both our filling station attendants and our controls. In fact, all three biomarkers of internal dose were higher in smokers and were correlated with the number of cigarettes smoked per day and during the work shift. The rho coefficients were particularly high for SPMA and urinary benzene.

Moreover, regression analysis showed a dependency of both SPMA and urinary benzene on the cigarette smoke, while only SPMA was dependent also on the airborne benzene measured during the environmental sampling procedure. These results differ from those reported for occupational exposure to low concentrations of airborne benzene, in which urinary benzene and SPMA showed a comparable dependency on airborne benzene and cigarette smoke (8). Therefore, urinary benzene seems to be more strongly affected by smoking habit than SPMA, which makes it a less valid biomarker of the internal dose for biological monitoring at the very low benzene concentrations prevailing nowadays.

Analyses of the ratio of urinary benzene to SPMA or *t,t*-MA made when studying benzene-toluene co-exposure provide one possible explanation for this result (table VI). In fact, while the results exclude the inhibitory effect of co-exposure to toluene on the metabolism of benzene described in some previous studies, they show that cigarette smoke could be a competitive inhibitor of the biotransformation of benzene (21, 22). This is probably due to the high quantities of chemical substances contained in the smoke, some of which may be metabolized through the same metabolic pathways as benzene, especially the oxidative CYP2E1 pathway (23). Therefore, cigarette smoke is a source of benzene that provokes an overall increase in the excretion of both benzene metabolites, as well as the unmodified form, although the latter has a proportionally greater effect. This is particularly evident in conditions of exposure to very low doses of the toxicant. For this reason, cigarette smoke will tend to have a proportionally greater influence on the urinary benzene concentrations than on *t,t*-MA and SPMA. Instead, as described above, it has no influence on the measured airborne benzene concentrations. Accordingly, urinary benzene will show a lack of dependency on airborne benzene in conditions of exposure to less than 66 µg/m^3, whereas for exposure to low concentrations like those analyzed in our previous study, the effect of cigarette smoke was not sufficient to offset the dependency of urinary benzene on airborne benzene.

The results of the present research showed that, at very low benzene concentrations, cigarette smoke is able to influence the SPMA and urinary benzene concentrations not only according to the number of cigarettes smoked but also to the time between the last cigarette and urine collection to determine these urinary biomarkers, since both results are negatively correlated with these factors. Moreover, the lower the exposure the higher the influence of cigarette smoking, that yields largely comparable rho coefficients for SPMA (rho=-0.43) and urinary benzene (rho=-0.45). The influence of the time between the last cigarette and urine collection has resulted higher in the two hours before urine collection (data not showed) according to the rapid excretion particularly of urinary benzene.

In conclusion, for exposure to very low benzene concentrations like those observed in the present study, uri-

nary benzene showed poor validity as a biomarker of occupational exposure to the toxicant, whereas its validity was conserved for SPMA, even if a smoking habit has a strong influence on both indexes. In addition, *t,t*-MA shows a minor validity as a biomarker of internal dose due to its lesser specificity for benzene. In any case, it seems that when using SPMA and urinary benzene to assess exposure to benzene a correct interpretation of the results must take into account not only the number of cigarettes smoked before urine collection but also the time between the last cigarette before collection. Alternatively, subjects should be asked to abstain from smoking for at least two hours before urine collection. Finally, information obtained by environmental sampling seems to be an essential part of any assessment of occupational exposure in worker categories exposed to very low concentrations of benzene, and a necessary complementary factor to be taken into account when interpreting the results of biological monitoring.

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