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Influence of tobacco smoke on urinary trans, trans-muconic acid levels evaluated by cotinine analysis in urine in a population from southern of Minas Gerais, Brazil

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INFLUENCE OF TOBACCO SMOKE ON URINARY TRANS,TRANS-MUCONIC ACID LEVELS EVALUATED BY COTININE ANALYSIS IN URINE IN A POPULATION FROM SOUTHERN OF MINAS GERAIS, BRAZIL

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SUMMARY

Trans,trans-muconic acid (ttMA) is one of the metabolites of benzene excreted in urine that has proven to be a suitable biomarker for benzene exposure <1ppm. However, it is not a specific biomarker for benzene exposure because the values can be influenced by other factors, such as diet, tobacco smoke and toluene co-exposure. The aim of this study was to verify the influence of tobacco smoke on urinary ttMA levels using a specific biomarker of tobacco smoke exposure, urinary cotinine, as well as to assess the ttMA variability in Brazilian subjects, smokers and non-smokers. The ttMA was analyzed by liquid chromatography with an ultraviolet detector following solid phase extraction, and urinary cotinine was analyzed by gas chromatography with a nitrogen-phosphorus detector after liquid-liquid extraction. The mean ± standard deviation of ttMA was 1.12 ±1.07μg·ml^{-1} (median 0.80μg·ml^{-1}) in smokers and was 0.22 ±0.21μg·ml^{-1} (median 0.17μg·ml^{-1}) in non-smokers. The concentration of urinary cotinine in smokers was 2.54 ±0.52μg·ml^{-1} (median 2.05μg·ml^{-1}); it was below the limit of quantification (0.01μg·ml^{-1}) in non-smokers. In the present study, the urinary ttMA and cotinine concentrations were determined by validated analytical methods and correlation between the biomarkers were observed (r= 0.41). While the urinary ttMA metabolite presents advantages in the simplicity of analysis, it had a relatively high background level in the non-smoker group, and there was large inter-individual variability in smokers.

Introduction

Benzene has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (1987), and it is a ubiquitous environmental pollutant as well as an important industrial chemical. It is used in the manufacture of a wide variety of consumer products and is present in gasoline at concentrations up to 1% (v/v) in many countries (WHO, 2000). Due to its industrial importance, benzene is not likely to be eliminated from ambient and occupational environments (Carrieri *et al*., 2006). However, exposure to benzene is carefully monitored due to its hematopoietic toxicity and leukemogenic properties (Ducos *et al*., 1992). The threshold limit values (TLV) of benzene in work places have been lowered in most industrialized countries. Brazil established technological reference values (TRV) of 1 and 2.5ppm for petrochemical plants and refineries, respectively (MLE, 1995). Biological monitoring of benzene exposure involves the determination of the concentration of benzene or its metabolites in the biological fluids of exposed subjects (Carrieri *et al*., 2006).

Trans,trans-muconic acid (ttMA) and S-phenylmercapturic acid (S-PMA) are minor metabolites of benzene that are excreted in urine and have been demonstrated to be suitable biomarkers for benzene exposure <1ppm (Boogard and Van Sittert, 1995; Pezzagno *et al*., 1999). Urinary S-PMA is considered to be a specific biomarker of benzene exposure (Melikian *et al*., 1999), and the analytical methods for its determination involve chromatography coupled to mass spectrometry (Melikian *et al*., 1999; Waidyanatha *et al*., 2004).

The biomarker ttMA is also routinely used to assess benzene exposure. In Brazil, ttMA use is recommended by the Ministry of Labor and Employment (MLE, 2001) despite its relatively low specificity to benzene exposure; its values can be influenced by other factors such as diet (sorbic acid, a common antimycotic food additive, is partially metabolized into ttMA) (Negri *et al*., 2005; Carrieri *et al*., 2006), tobacco smoke (Melikian *et al*., 1993; Paula *et al*., 2003; Fustinoni *et al*., 2005; Menezes *et al*., 2008) and toluene co-exposure (Inoue *et al*., 1989).

The concentration of benzene in cigarette smokers is relatively high. A 30 cigarette/day smoker's personal daily uptake of benzene is between

KEYWORDS / Benzene / Cotinine / Tobacco smoke / Trans,Trans-muconic Acid /

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INFLUENCIA DEL HUMO DE TABACO EN LOS NIVELES URINARIOS DE ÁCIDO TRANS,TRANS-MUCÓNICO EVALUADOS POR ANÁLISIS DE COTININA EN ORINA DE UNA POBLACIÓN DEL SUR DE MINAS GERAIS, BRASIL

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RESUMEN

El ácido trans,trans-mucónico (AttM) es uno de los metabolitos del benceno excretados en la orina que ha probado ser un buen marcador de la exposición al benceno <1ppm. Sin embargo, no es un buen marcador específico para benceno porque sus valores pueden ser influenciados por otros factores, tales como dieta, humo de tabaco y exposición al tolueno. El propósito de este estudio fue verificar la influencia del humo del tabaco en los niveles de AttM urinario utilizando un marcador específico de la exposición al humo del tabaco, la cotinina urinaria, así como determinar la variabilidad del AttM en sujetos fumadores y no fumadores brasileros. El AttM fue medido por cromatografía con detector ultravioleta tras extracción en fase sólida, y la cotinina urinaria fue determinada por cromatografía con un detector de fósforo y nitrógeno, tras extracción líquido-líquido. El promedio ± desviación estándar de AttM fue de 1,12 ±1,07μg·ml^{-1} (mediana de 0,80μg·ml^{-1}) en fumadores y de 0,22 ±0,21μg·ml^{-1} (mediana de 0,17μg·ml^{-1}) en no fumadores. La concentración urinaria de cotinina en fumadores fue de 2,54 ±0,52μg·ml^{-1} (mediana de 2,05μg·ml^{-1}), mientras que en no fumadores estuvo por debajo del límite de detección (0,01μg·ml^{-1}). En el presente estudio, las concentraciones urinarias de AttM y cotinina fueron determinadas por métodos analíticos validados y se encontró correlación (r= 0,41) entre los marcadores. Aunque el metabolito urinario del AttM presenta ventajas por la simplicidad del análisis, tuvo un nivel relativamente alto en el grupo de no fumadores y hubo una gran variabilidad entre individuos fumadores.

INFLUÊNCIA DA FUMAÇA DO TABACO NOS NÍVEIS URINÁRIOS DE ÁCIDO TRANS,TRANS-MUCÔNICO AVALIADOS POR ANÁLISE DE COTININA URINÁRIA EM UMA POPULAÇÃO DO SUL DE MINAS GERAIS, BRASIL

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RESUMO

O ácido trans,trans-mucônico (AttM) é um dos metabólitos do benzeno excretados na urina que tem provado ser um bom marcador da exposição ao benzeno <1ppm. No entanto, não é um bom marcador específico para benzeno porque seus valores podem ser influenciados por outros fatores, tais como dieta, fumaça de tabaco e exposição ao tolueno. O propósito deste estudo foi verificar a influência da fumaça do tabaco nos níveis de AttM urinário utilizando um marcador específico da exposição a fumaça do tabaco, a cotinina urinária, assim como determinar a variabilidade do AttM em sujetos fumadores e não fumadores brasileiros. O AttM foi medido por cromatografia com detector ultravioleta após extração em fase sólida, e a cotinina urinária foi determinada por cromatografia com um detector de fósforo e nitrogênio, após extração líquido-líquido. A média ± desviação estándar de AttM foi de 1,12 ±1,07μg·ml^{-1} (média de 0,80μg·ml^{-1}) em fumadores e de 0,22 ±0,21μg·ml^{-1} (média de 0,17μg·ml^{-1}) em não fumadores. A concentração urinaria de cotinina em fumadores foi de 2,54 ±0,52μg·ml^{-1} (média de 2,05μg·ml^{-1}), enquanto que em não fumadores esteve por debaixo do límite de detecção (0,01μg·ml^{-1}). No presente estudo, as concentrações urinarias de AttM e cotinina foram determinadas por métodos analíticos validados e se encontrou correlação (r= 0,41) entre os marcadores. Ainda que o metabolito urinario do AttM apresenta vantagens pela simplicidade del análise, teve um nivel relativamente alto no grupo de não fumadores e houve uma grande variabilidade entre individuos fumadores.

1500 to 1800μg (Melikian *et al*., 1999) and can be a confounding factor in ttMA values in occupational exposure monitoring. The reported levels of urinary ttMA are higher in smokers than in non-smokers (Lee *et al*., 1993; Ruppert *et al*., 1995; Scherer *et al*., 1995; Paula *et al*., 2003; Wiwanitkit *et al*., 2005). Measurement of cotinine, a metabolite of nicotine, in biological fluids has been used as a biological indicator of the internal dose of tobacco (Scherer *et al*., 1995; Fustinoni *et al*., 2005; Wiwanitkit *et al*., 2005).

The previous papers, that discussed the correlation of the smoking habit and ttMA excretion in a Brazilian population, evaluated tobacco smoke exposure by a questionnaire about the number of cigarettes smoked and did not quantify the internal dose of cigarette components (Paula *et al*., 2003; Menezes *et al*., 2008). Determination of cotinine and ttMA urinary in the biological monitoring of benzene exposure could allow for improvements in determining the tobacco smoke contribution to urinary levels of ttMA, which, in turn, could refine our ability to assess occupational benzene exposure.

The aim of this study was to investigate the influence of tobacco smoke on urinary ttMA levels by studying the correlation between its levels and the urinary cotinine, which were compared in a Brazilian population of smokers and non-smokers. In addition the effect of individual factors in the biomarkers urinary excretion was evaluated.

Methods

Reagents and chemicals

Cotinine (~98% purity, lot Nº 055k4053), lidocaine (~98% purity; lot Nº 162008) and trans,trans-Muconic acid (ttMA; ~98% purity) were purchased from Sigma-Aldrich (St. Louis, USA). Analytical grade isopropyl alcohol, chloroform and Methanol (HPLC grade) were purchased from Vetec (Rio de Janeiro, Brazil), and sodium hydroxide from Labsynth (São Paulo, Brazil). Glacial acetic acid p.a. was obtained from Furlab (Campinas, Brazil). A SAX cartridge filled with 500mg of strong anionic exchange silica was purchased from Supelco (USA). Throughout the study, water was obtained from a Milli-Q system from Millipore (São Paulo, Brazil).

Stock solutions of cotinine and lidocaine (internal standard) were prepared in isopropyl alcohol at 1mg·ml^{-1} and

stored at -20°C, protected from light. The solutions were used for at least one month. Working solutions were freshly prepared in isopropyl alcohol immediately before analysis. A stock solution of ttMA was prepared in methanol at 1mg·ml^{-1} and stored at -20ºC, protected from light. This stock solution was used for only one month, and working solutions were freshly prepared.

Urine samples

Urine samples were collected from 109 Brazilian subjects. These included 82 active smokers and 27 non-smokers who have no known history of benzene exposure. The individuals lived in the south of Minas Gerais, in a region considered as having low rates of pollution. Characteristics of the smoker and non-smoker subjects are shown in Table I. This study was approved by the Ethics Committee of the Federal University of Alfenas. Informed consent was obtained from each volunteer as well as a questionnaire containing information about their eating habits, number of cigarettes/day and possible occupational exposure to benzene. None of the non-smokers selected in this study lived or worked with smokers. Samples were collected in two periods, in the morning (first urine/day) and at night (last urine/day), in polyethylene urine containers and frozen for storage at -20ºC until analysis. Density was measured at each sampling time with the Atago® refractometer for standardization of ttMA and cotinine levels.

Instrumentation and chromatographic conditions

A Clarus 400 model gas chromatograph equipped with a nitrogen-phosphorus detector (GC-NPD) (Perkin Elmer; Connecticut, USA) with Totalchrom Workstation Software (Connecticut, USA) was used for cotinine analysis.

TABLE I
CHARACTERISTICS OF THE POPULATION STUDIED

Parameters		Smokers Mean (median)	Non smokers Mean (median)
Nunmber of subjects		82	27
Age		35.9 (38.0)	27.4 (23.0)
Gender	Female	41	15
	Male	41	12
Cigarettes/day	1-10	36	
	>10	46	

Chromatographic analysis was performed with a ZB-1 column (Phenomenex; 100% polydimethylsiloxane; 30m×0.53mm i.d.; 5µm film thickness). Nitrogen was used as the gas carrier at a pressure of 4.3psi. A 1µl injection volume was manually injected using a splitless mode with an injector temperature of 260ºC. The oven temperature was programmed from a starting temperature of 180ºC to increase by 30ºC/min to 250ºC for 0.5min, by 15ºC/min to 259ºC for 0.1min and by 0.1ºC/min to 260ºC. The detector temperature was 280ºC. The total run time was 13.4min.

Urinary ttMA determination was performed by liquid chromatography (HPLC) using a Shimadzu pump model LC-AT VP with an SPD-10 UV detector at 264ηm and the Phenomenex analytical RP 18 column (15cm×4.6mm×5µm) maintained at 30ºC. The mobile phase was acetic acid 1%: methanol (90:10, v/v) with a flow rate of 1ml·min^{-1}.

Sample preparation

The levels of cotinine were determined according to the method previously described by Ceppa *et al.* (2000) with some modifications. Before extraction, samples were thawed and equilibrated to room temperature. For cotinine analysis, urine (5.0ml) was placed in a 15ml glass centrifuge tube. One ml of sodium hydroxide (5mol·l^{-1}), 50µl of lidocaine internal standard (100µg·ml^{-1}) and 5.0ml of chloroform were added to the tube and mixed during 15min in a bench top shaker. After centrifugation for 15min at 840g, the aqueous layer was discarded, and a 4.5ml aliquot of the organic phase was transferred into conical glass tube. The extract was evaporated under a stream of nitrogen in a 40ºC water bath. The residue was reconstituted in 50µLl isopropyl alcohol.

The levels of ttMA were determined according to the method developed by Ducos *et al.* (1992), with some modifications. Briefly, urine (0.25ml) was applied to a SAX cartridge preconditioned with 1.5ml methanol and water. The cartridge was washed with 2.0ml of a 1% aqueous glacial acetic acid solution; ttMA was then eluted with 1.0ml of a 10% aqueous glacial acetic acid solution. The eluate was analyzed by HPLC, with an injection volume of 100µl.

Validation parameters

The method for measuring cotinine was validated and was linear in the range of 0.01 to 6µg·ml^{-1}, with determination coefficients of 0.9969. The limit of quantification (LOQ) was 0.0lµg·ml^{-1}. Intra- and inter-assay precision analyses produced relative standard deviations lower than 8.5% at low, medium and high levels. The efficiency of extraction was greater than 88.5%. Ruggedness was verified by varying the following parameters: sample volume, NaOH concentration, agitation level in the vortex, time in bench top shaker, centrifugation time, detector temperature and gas carrier pressure. For the ttMA method, previously validated in the Laboratory of Toxicological Analysis (Menezes *et al.*, 2008), results demonstrated linearity in the range of 0.01-10µg·ml^{-1} with a determination coefficient of 0.999, a LOQ of 0.01µg·ml^{-1}and satisfactory precision with relative standard deviations <11%.

Statistical methods and data analysis

Non-parametric tests (Mann-Whitney for difference of medians in two groups, Kruskal-Wallis for difference of medians in three or more groups and Spearman's test for correlation) were used. All statistical analyses were conducted using Bioestat 4.0 statistical software and Excel software. Differences were deemed to be statistically significant at $p \leq 0.05$.

Results and Discussion

Large inter-individual variability in the urinary concentrations of the metabolites was observed among the 82 smokers and 27 non-smokers included in the study. Thus, the values were represented as density-corrected concentrations, which is a common practice for expressing urinary metabolites when only spot urine samples are available (ACGIH, 2006). Authors have described ttMA and cotinine values using the urinary density for normalization (Lee *et al.*, 1993; Melikian *et al.*, 1993; Ruppert *et al.*, 1995).

The distribution of ttMA was not normal, so non-parametric tests were used in further analyses of the data. Similar treatment of data was reported in the study by Paula *et al.* (2003). The characteristics of the smoker and non-smoker subjects are presented in Table I. A significant correlation between the two biomarkers ($r = 0.41$, $p = 0.0012$) was found, in agreement with the literature (Melikian *et al.*, 1993; Scherer *et al.*, 1998; Fustinoni *et al.*, 2005). In Fig-

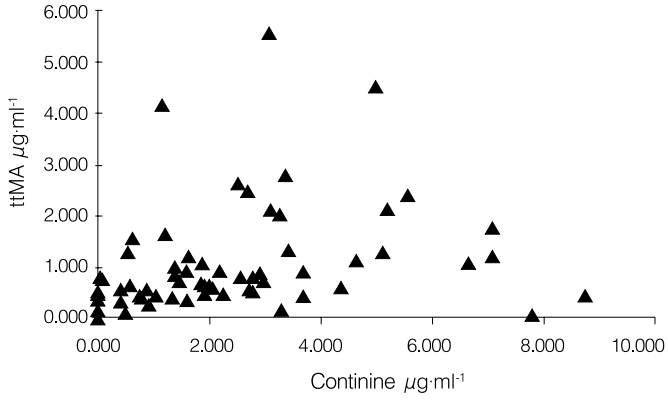

Figure 1. Relationship between urinary concentrations of ttMA in smokers (n= 82) and non-smokers (n= 27) (r= 0.41, p= 0.0012).

TABLE II
MEAN URINARY CONCENTRATIONS OF TRANS,TRANS-MUCONIC ACID AND COTININE (± STANDARD DEVIATION) IN SMOKERS AND NON-SMOKERS

		Number of samples		ttMA (μg·ml^{-1}) mean ±SD (median)		Cotinine (μg·ml^{-1}) mean ±SD (median)	
		Smokers	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers
Subjects (n)		82	27	1.12 ±1.07 (0.80)	0.22 ±0.21 (0.17)	2.54 ±0.52 (2.05)	0.01 ±0.004 (0.01)
Gender	Female	41	15	1.34 ±1.37 (0.94)	0.28 ±0.20 (0.24)	3.33 ±2.32 (2.59)	0.01 ±0.002 (0.01)
	Male	41	12	0.91 ±0.56 (0.72)	0.16 ±0.21 (0.024)	1.89 ±1.30 (1.84)	0.01 ±0.005 (0.01)
Period	Morning	82	27	1.31 ±1.30 (0.77)	0.25 ±0.17 (0.25)	2.96 ±2.24 (2.55)	0.01 ±0.001 (0.01)
	Night	82	27	0.96 ±0.81 (0.90)	0.20 ±0.24 (0.071)	2.31 ±1.55 (1.81)	0.01 ±0.005 (0.01)
Cigarettes/ day	1-10	36	0	0.84 ±0.64 (0.63)		2.25 ±1.08 (1.47)	
	>10	46	0	1.35 ±1.29 (0.82)		2.91 ±1.93 (2.73)	

ure 1, urinary ttMA and cotinine values measured in the same sample are plotted for both the smoker and non-smoker groups.

As shown in Table II, the mean concentration ±standard deviation (SD) of ttMA in smokers was 1.12 ±1.07μg·ml^{-1}, with a median concentration of 0.80μg·ml^{-1}. In non-smokers, the mean concentration was 0.22 ±0.21μg·ml^{-1}, and the median concentration was 0.17μg·ml^{-1}. The urinary ttMA concentrations reported by Lee *et al*. (1993) ranged from 0.03 to 0.33μg·ml^{-1} (mean= 0.13μg·ml^{-1}) for non-smokers (n= 23) and from 0.03 to 0.77μg·ml^{-1} (mean 0.25μg·ml^{-1}) for smokers (n= 35). Mean ttMA concentrations in smokers were significantly higher than in non-smokers, although the authors noted that there was some overlap in ttMA levels between smokers and non-smokers. Fustinoni *et al*. (2005) obtained median ttMA concentrations of 0.07μg·ml^{-1} for non-smokers (n= 36) and 0.19μg·ml^{-1} for smokers (n=13): according to these authors, following cigarette consumption, the levels of urinary biomarkers were systematically higher, with the exception of subjects from Milan, which did not display differences in metabolite levels in smokers compared to non-smokers. Gobba *et al*. (1997) proposed the hypothesis that the ability to metabolize benzene to this metabolite significantly varies among individuals in the general population, and two different groups exist: poor and efficient ttMA metabolizers.

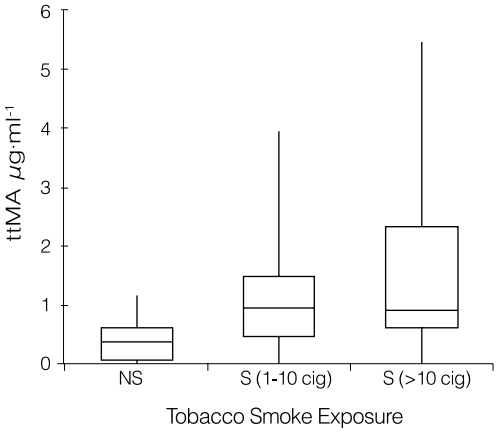

Figure 2. Multiple comparisons of urinary ttMA concentrations in the non-smoker and smoker groups (NS: non-smokers; S: smokers). The horizontal line in the box represents the median. Significant differences were observed among NS and S subgroups: p= 0.0002 (1-10 cigarettes/day) and p<0.0001 (>10 cigarettes/day); Kruskal-Wallis test.

The mean concentration ±SD of urinary cotinine in smokers was 2.54 ±0.52μg·ml^{-1}, and the median was 2.05μg·ml^{-1}. In all non-smoker samples, urinary cotinine concentrations were less than the LOQ (0.01μg·ml^{-1}). The values obtained in the present study are in agreement with those reported in the literature. The concentrations of urinary cotinine reported in the literature range from 0.073 to 6.68μg·ml^{-1} for smokers (Scherer *et al*., 1995; Oddoze *et al*., 1998; Voncken *et al*., 1998; Ji *et al*., 1999; Man *et al*., 2006).

The levels of urinary ttMA in smokers were significantly higher than those determined in non-smokers (p= 0.0061), and the same trend was observed for urinary cotinine (p<0.0001). No significant difference was observed between median urinary ttMA and the number of cigarettes consumed (p= 0.2830). These results are in agreement with those obtained by Paula *et al*. (2003) and Menezes *et al*. (2008). However, the urinary cotinine concentrations did display significant difference between the two subgroups (p= 0.0456). The multiple comparisons of ttMA concentrations in non-smoker and smoker groups are presented in Figure 2.

Volunteers in this study were carefully selected from a common geographical location with uniform environmental conditions, however, a large degree of variability in ttMA levels was observed among individuals. The applicability of this biomarker for measuring low level benzene exposure may be limited because of confounding factors such as diet. Sorbic acid, a widely-used food preservative, is also a precursor of ttMA. Analysis of the questionnaires revealed that most volunteers consumed foods containing sorbic acid, such as ketchup, processed sauces and soft drinks, which could contribute to the variability observed in urinary ttMA values. However, further studies are needed to confirm this hypothesis.

Ruppert *et al*. (1995) found that intake of sorbic acid would lead to an excretion of 0.01-0.04μg·ml^{-1} of ttMA and, thus, could interfere with levels predicted to occur following low-level environmental benzene exposure. Scherer *et al*. (1998) concluded that biomonitoring of benzene exposure using urinary ttMA appears to be possible only if the ingestion of dietary sorbic acid is taken into account. Marrubini *et al*. (2002) showed the dose-response relationship between sorbic acid administration and ttMA excretion; the data support the

conclusion that this metabolite is not suitable for biomonitoring of low levels of benzene exposure. Weaver *et al.* (1996) found that sorbic acid has the potential to cause substantial interference with ttMA excretion.

In this study, the statistical analyses showed no significant difference among ttMA urinary excretion in male and female smokers ($p>0.05$), which is in agreement with findings by Paula *et al*. (2003). In constrast, Melikian *et al*. (2002) suggested that women excrete more metabolites than men for the same levels of benzene exposure. A significant correlation ($r= 0.33$, $p= 0.0099$) was observed between the levels of excreted ttMA and age. This was also reported by Paula *et al*. (2003) in a group not exposed to benzene occupationally (results not shown). However, no significant correlation was observed among urinary ttMA and age in the smoker group. The correlation was demonstrated in relation to the levels of urinary cotinine and age ($r= 0.49$, $p<0.05$).

No significant differences were observed in the levels of ttMA and cotinine from samples collected at different times of the day (i.e., morning and night; $p>0.05$). This result is in contrast to the findings of Lee *et al*. (2005) obtained from factory workers without solvent exposure (21 non-smokers and 26 smokers) and students and hospital staff (14 non-smokers), who displayed urinary ttMA levels that were lowest in the morning, highest in the afternoon and then low again at night (results not shown). According to the results obtained by Martins and Siqueira (2004) biological monitoring for individuals occupationally exposed to benzene is most effective when urine samples are taken at the end of the work shift (results not shown).

The results obtained in the present study are not sufficient to clarify the magnitude of the inf luence of tobacco smoke on ttMA excretion, and to determinate all possible sources of interference with urinary ttMA levels. Further studies with different exposure conditions are necessary to confirm the findings. On the other hand, from a toxicological point of view, the correlation between the biomarkers was tested experimentally in a Brazilian population. As benzene is a universal contaminant, authors have discussed the urgency of environmental and biological evaluation, in both exposed and non-exposed populations, using criteria that permit the accurate interpretation of the data (Costa and Costa, 2002; Machado *et al*., 2003).

Conclusions

The urinary ttMA and cotinine concentrations were determined by validated analytical methods and correlation between the biomarkers was observed. While the urinary ttMA metabolite presents advantages in the simplicity of analysis, it presented a relatively high background level in the non-smoker group, and there was large inter-individual variability in smokers. It could be recommended that if urinary ttMA is chosen as a benzene biomarker, the diet should be controlled, and in smokers the urine sampling should be performed in pre and post-shift samples, with simultaneous cotinine analysis. These parameters could provide a viable method for assessing exposure to low concentrations of benzene.

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