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trans,trans-Muconic acid, a reliable biological indicator for the detection of individual benzene exposure down to the ppm level

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Summary. *trans,trans*-Muconic acid (2,4-hexadienedioic acid) (*t,t*-MA) is a minor benzene metabolite which can be used as a biological indicator for benzene exposure. The purpose of the study was to evaluate the limits of use of *t,t*-MA for detection and quantification of occupational exposures to benzene, particularly on an individual scale, phenol being used as the metabolite of reference. A simple and sensitve method previously described by the authors was carried out to analyse *t,t*-MA in 105 end-of-shift urinary samples from 23 workers exposed to benzene used as an extraction solvent for "concretes" recovery in the perfume industry. Good correlations were found between atmospheric benzene and both metabolites (uncorrected or corrected for creatinine) or between the metabolites themselves, with correlation coefficients from 0.81 to 0.91 ($P < 0.0001$). Correlation- coefficients were not improved after correction for creatinine. The overall individual benzene exposure range, median, and arithmetic mean were respectively 0.1–75, 4.5, and 9.0 ppm with corresponding *t,t*-MA excretion of 0.1–47.9, 5.2 and 8.9 mg/l (uncorrected) and phenol excretion of 1.4–298, 30.9, and 42.2 mg/l (uncorrected). In the control group (145 determinations for *t,t*-MA and 76 for phenol from 79 individuals) the range, median, and arithmetic mean were respectively < 0.04–0.66, 0.08, and 0.13 mg/l (uncorrected *t,t*-MA) and 1.5–42.0, 9.85 and 11.3 mg/l (uncorrected phenol). *t,t*-MA was far more specific than phenol and could be easily and practically used to estimate with a given probability the upper or lower corresponding benzene concentrations down to around the ppm level. Biological exposure indices for benzene exposure to 10, 5, or 1 ppm could be set at 10, 5, or 1 mg *t,t*-MA/l (uncorrected).

Key words: Benzene – Biological monitoring – *trans, trans*-Muconic acid – Individual exposures – BEIs proposals

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Introduction

Occupational exposure to benzene, a major compound for the organic chemical industry, is under particular supervision due to its hematopoietic toxicity and leukemogenic properties. Atmospheric thresholds, still different in many countries, are being progressively lowered towards 5 or 1 ppm. In their last Notice of Intended Change the ACGIH even proposed 0.1 ppm as a new TLV-TWA (ACGIH 1990–1991). Therefore, a more reliable and sensitive biological exposure index (BEI) is needed for benzene than 50 mg/l of urinary phenol, which is the most probable bioequivalent of around 10 ppm benzene [14]. Other authors [9, 12] have shown the potential of *trans,trans*-muconic acid (2,4-hexadienedioic acid) as a substitute for phenol, which is considered not specific enough at low levels of benzene, but it was concluded [12] that *trans,trans* muconic acid (*t,t*-MA) could only show benzene exposures at 6 or 7 ppm on a group basis.

Very recently, Bechtold et al. [2], despite the limited size of their sample of exposed workers, considered that individual exposures of as little as 4–5 ppm could be easily distinguished from nonexposure and suggested that *t,t*-MA might be useful to as little as 1 ppm benzene. We agree with them, since in our previous work we already expected a much lower limit of detection using a simple, sensitive, and reliable analytical procedure designed to measure *t,t*-MA down to around 0.05 mg/l [6]; this value is approximately a tenth of the amount of *t,t*-MA or its equivalent found in the most heavily concentrated urine of unexposed people.

In order to compare the advantages of *t,t*-MA and phenol in these conditions, the latter was used as the reference at different atmospheric concentrations of benzene.

The aim of this work is to determine the limits of the use of *t,t*-MA in assessing benzene exposure by studying the correlation between benzene levels and the urinary concentrations of both metabolites, which are in addition compared to those of unexposed people.

Materials and methods

Factories. Three factories were selected according to their use of benzene as the extraction solvent for producing raw perfumery products from vegetable or animal origin. Among hydrocarbon solvents, benzene was widely used because of its extractive properties.

Although there is a trend to replace benzene by less toxic solvents, benzene is still used in batteries to extract resinoids (incense, myrrh, benzoin, galbanum, tolu, etc), mosses (tree moss, Yugoslav oakmoss, etc.), hay, cereal bran, lavender, lavandin, cistus, tonka beans, fenugreek, castoreum, civet, and so on. After evaporation of the benzene, the extracts, called "concretes", are further extracted with ethanol to discard insoluble matter undesirable for the production of perfumes. The end product, called an "absolute", can be liquid or semisolid.

The three factories, taken together, had a wide range of potential atmospheric benzene concentrations, from less than 1 ppm to 100 ppm or more.

Exposed workers. Twenty-three workers were selected in the three factories. They were all involved in the extraction procedure, benzene evaporation, and recovery of the concretes. The operations being not the same every day, and jobs and working habits being different in each of the three factories, the exposure profiles were considered very diverse and mean exposure levels independent.

As one the main goals of this work was to study the overall variability of the biological data resulting from exposure to various benzene concentrations, *t,t*-MA and benzene measurements in the same workers were repeated over the week. After analysis of biological and atmopsheric samples for each worker, urinary data were considered to be mainly linked to benzene concentration. Therefore, all biological data were treated as if they were independent.

Coexposures able to significantly modify the metabolism of benzene in the workers were the exception: only one extractor was near to a synthesis unit which could be considered a source of possible metabolic competitors (toluene and xylene were used among several other organic compounds). All the workers studied were asked to wear a personal air sampling device with a sorbent tube and to avoid any cutaneous contact. Their urine was collected daily at the beginning and end of the work shift for a working week, and kept frozen at −20°C before analysis.

Some data are lacking for the study of the correlation of benzene to metabolites, due to either use of cartridge respirators or absence from work. In one case only, the data were voluntarily discarded because the worker had a high exposure immediately before the end of shift.

Control group. To determine the normal background levels of *t,t*-MA, which are often low and sometimes difficult to evaluate, 79 individuals not occupationally exposed to benzene were selected from the administrative staff of the three factories and the work force of four other plants (workers and administrative staff not exposed to benzene); 145 determinations of *t,t*-MA in end-of-shift urine samples were used to take into account a possible effect of nutrition on the excretion of this metabolite, which is also a minor metabolite of sorbic acid [6, 21], a widely used food preservative, and has been shown to occur in urine of healthy individuals [15]. Traces of benzene from smoking are, we believe, too low to be accurately detected via urinary *t,t*-MA; calculations based on the supposition of a maximum benzene daily intake of 2 mg in a heavy smoker and on both our previous results and metabolism rate [12] along with analyses of some samples from smokers and non-smokers confirm this statement. Therefore, smoking habits were considered a nonconfounding factor.

Phenol, for which a great amount of data already exists, was determined by analysis of only 76 end-of-shift voids (17 different workers in four factories) to relate our data to those published.

Urinary t,t-MA. The method of determination of urinary *t,t*-MA was described in detail in a previous paper [6]. Hippuric acid, which is always present at generally high concentrations in urinary extracts, was used as the reference component ($t_r \simeq 20$ min) for a better identification of *t,t*-MA at low levels.

Urinary phenol. The urinary concentration of phenol was determined according to the general scheme of the analytical method of the National Institute of Occupational Safety and Health (NIOSH) [18] with a modification of the concentration of the internal standard solution (nitrobenzene), which was set up at 6 mg/ml. *t,t*-MA and phenol concentrations were both uncorrected and corrected only for creatinine (Jaffe method) for the correlation study, as proposed by the NIOSH in its manual of analytical methods [18].

Atmospheric benzene. Personal sampling based on portable Gilian LFS pumps and SKC charcoal tubes (226-01), and GC analyses were performed according to the French AFNOR standard (NFX 43.251, July 1986).

Analytical procedures

Statistical analysis. Descriptive statistics, regression lines, and confidence limits were calculated using the SAS software (Statistical Analysis System, SAS Institute, Inc., Cary, N.C.). Calculations were done after logarithmic transformation on both the metabolites and benzene concentrations for two major reasons. First, log-normal distributions are considered to be better than normal distributions (or at least not worse) when the range of values is closed at the lower end but is open at the higher end, as for many materials in the body fluids [10]. Secondly, as the variability of the metabolite concentrations increases with the atmospheric benzene level, statistical inference using a simple linear regression is not valid. In particular, confidence intervals for low values of concentrations would be unreasonably large. These problems are solved by the log transformation, used more and more frequently in similar correlation studies [e.g. 3, 4, 7, 8, 13, 16, 19].

Results and discussion

Exposed workers

The results of the air and biological monitoring (end-of-shift) carried out on exposed workers in the three factories involved are summarized in Table 1.

In factory 1, some very highly polluting operations provided much higher concentrations than 75 ppm. However, the results were neither included in Table 1 nor used to calculate the regression line because the worker involved in these operations wore a cartridge respirator.

The distributions of benzene and both the metabolites, uncorrected and corrected for creatinine, were also tested according to the "univariate" procedure from SAS, using the Shapiro-Wilk statistic. They were all shown to fit a near log-normal distribution, especially all the data which did not come from a normal distribution. This finding is in agreement with the previous hypothesis. After logarithmic transformation, linear relationships were found between atmospheric concentrations of benzene and concentrations of its metabolites (*t,t*-MA and phenol uncorrected or corrected for creatinine): in all cases, there was a very significant correlation (Table 2). Since correlation coefficients were not improved by correcting the metabolite concentrations for creatinine, all further discussion of data and results will be essentially focused on the uncorrected data alone.

Table 1. Descriptive statistics for individual benzene concentrations, urinary *t,t*-MA, and phenol levels in end-of-shift urine samples of subjects from the three factories

	Factory 1		Factory 2		Factory 3		All	
Number of workers	8		8		7		23	
Number of sets of determinations	34		37		34		105	
Benzene (ppm)								
Range	2.4– 75		0.1– 3.6		2.3–11.8		0.1– 75	
Median	17.5		0.9		5.4		4.5	
Arithmetic mean	21.0		1.0		5.7		9.0	
Geometric mean	15.1		0.7		5.3		3.7	
Geometric standard deviation	2.5		2.3		1.5		4.4	
t,t-MA mg/l (mg/g creatinine)								
Range	1.2– 47.9	(0.8– 30.1)	0.1– 8.0	(0.1– 5.7)	0.7–19.5	(0.6–15)	0.1– 47.9	(0.1– 30.1)
Median	16.6	(8.4)	0.9	(0.7)	8.3	(6.0)	5.2	(3.3)
Arithmetic mean	17.1	(10.5)	1.4	(1.0)	10.0	(6.7)	8.9	(5.9)
Geometric mean	11.7	(7.3)	0.8	(0.6)	7.1	(5.4)	3.9	(2.8)
Geometric standard deviation	2.7	(2.6)	2.8	(2.6)	2.2	(2.1)	4.5	(2.8)
Phenol mg/l (mg/g creatinine)								
Range	5.4–298	(5.2–167)	1.4–48.2	(1.1–37.6)	17.5–86.7	(15.4–86.7)	1.4–298	(1.1–167)
Median	62.1	(33.8)	11.0	(7.8)	38.5	(32.0)	30.9	(21.2)
Arithmetic mean	71.6	(43.4)	14.3	(10.0)	43.0	(32.3)	42.2	(28.0)
Geometric mean	50.7	(31.9)	10.4	(8.0)	39.6	(29.9)	26.7	(19.2)
Geometric standard deviation	2.5	(2.3)	2.3	(2.0)	1.5	(1.5)	2.8	(2.5)

Table 2. Correlation coefficients of the linear relationships between the variables (log transformed; $n = 105$; $P < 0.0001$)

	t,t-MA (mg/l)	*t,t*-MA (mg/g creatinine)	Phenol (mg/l)	Phenol (mg/g creatinine)
Benzene (ppm)	0.90741	0.90475	0.81894	0.82755
t,t-MA (mg/l)	–	–	0.87309	–
t,t-MA (mg/g creatinine)	–	–	–	0.85740

The relationship between benzene and *t,t*-MA is better than that between benzene and phenol. In the latter case, the equation of the regression line (Table 3) gives, for a benzene exposure to 10 ppm, an urinary phenol excretion level of 47.5 mg/l, which is consistent with the ACGIH-BEI of 50 mg/g creatinine for a mean creatinine concentration of 1 g/l.

Control group

t,t-MA and phenol data for the control group are summarized in Table 4. When *t,t*-MA concentrations were not detectable, the limit of detection (approximately 0.04 mg/l) was divided by 2 for further calculations on all the data [11, 20].

As in the case of the exposed workers, the distributions were found to be rather log-normal than normal; the geometric means for both *t,t*-MA and phenol, respectively 0.086 and 9.93 mg/l, were close to the observed medians (0.08 and 9.85 mg/l). The distribution of *t,t*-MA concentrations was consistent with the data of Inoue et al. [12]: 64% were below 0.1 mg/l, compared to 52% in the present study (67% were ≤ 0.1 mg/l), where the 95th percentile was 0.4 mg/l. However, we never found concentrations higher than 0.7 mg/l, whereas their highest value was 2.0 mg/l.

Specificity of t,t-MA compared to phenol

The concentrations of both metabolites in the control group were compared with the calculated values from the regression lines at different benzene concentration levels. Table 5 shows the percentage of concentrations that exceeded these values. It can be seen that all *t,t*-MA concentrations were lower than the value corresponding to 1 ppm of benzene, while the same result for phenol concentrations was only valid at 10 ppm.

Estimation of individual exposure

The regression line between the logarithmic concentrations of atmospheric benzene and urinary *t,t*-MA can be used to estimate most likely exposures through measurements of this metabolite. Furthermore, assuming log-nor-

Table 3. Coefficients of the linear equations of the regression lines ($\log y = a + b \log x$; $n = 105$)

y	x	a (Intercept)	b (Slope)	Mean square error
Benzene (ppm)	t,t-MA (mg/l)	0.040853	0.891456	0.07359
Benzene (ppm)	Phenol (mg/l)	−1.092684	1.163256	0.13723
t,t-MA (mg/l)	Benzene (ppm)	0.066541	0.923651	0.07325
Phenol (mg/l)	Benzene (ppm)	1.099917	0.576541	0.06802

Table 4. Descriptive statistics for t,t-MA and phenol in the control group

Variable	Number of determinations	Range (mg/l)	Median (mg/l)	Arithmetic mean (mg/l)	Geometric mean (mg/l)	Geometric standard deviation
t,t-MA	145	< 0.04– 0.66	0.08	0.13	0.086	2.5
Phenol	76	1.5 –42.0	9.85	11.3	9.93	1.6

Table 5. Specificity of t,t-MA compared with phenol

Benzene (ppm)	0.1	0.5	1	5	10
Corresponding t,t-MA concentrations ($C_{t,t\text{-}MA}$) (mg/l)	0.14	0.61	1.17	5.15	9.78
Percentage of controls with excretion levels higher than $C_{t,t\text{-}MA}$	32.4	0.7	0	0	0
Corresponding phenol concentrations (C_{phenol}) (mg/l)	3.3	8.4	12.6	31.8	47.5
Percentage of controls with excretion levels higher than C_{phenol}	97.3	75.0	19.7	3.9	0

mal distributions, it can be stated, on an individual scale and with a certain probability, that the estimated benzene concentration is higher or lower than a given value. For example, if the measured value of t,t-MA is higher than 17.8 mg/l (~ 18 mg/l), the benzene level will exceed 5 ppm – the current French VME (mean exposure value) – with a probability of 95% (Fig. 1). On the other hand, this VME will not be exceeded, with the same probability, when excretion levels are lower than 1.8 mg/l (~ 2 mg/l).

Similarly, t,t-MA excretions higher than 3 mg/l or lower than 0.3 mg/l could be used to determine individual exposures respectively above or below 1 ppm, which is a critical level below which there is no need for legal check-up more than once a year in France (Décret no. 86-269, 1986). However, it should be kept in mind that, if the t,t-MA concentrations can be considered as belonging to an unexposed population (roughly lower than 0.4 mg/l, which is the 95th percentile of the controls), the lower confidence limit can no longer be used to determine minimum exposures to benzene.

More generally, from a t,t-MA concentration, even low, an upper limit below which a worker has been exposed can be deduced with a given probability; for practical purposes this limit can be conveniently expressed as equivalent to an atmospheric exposure to benzene even

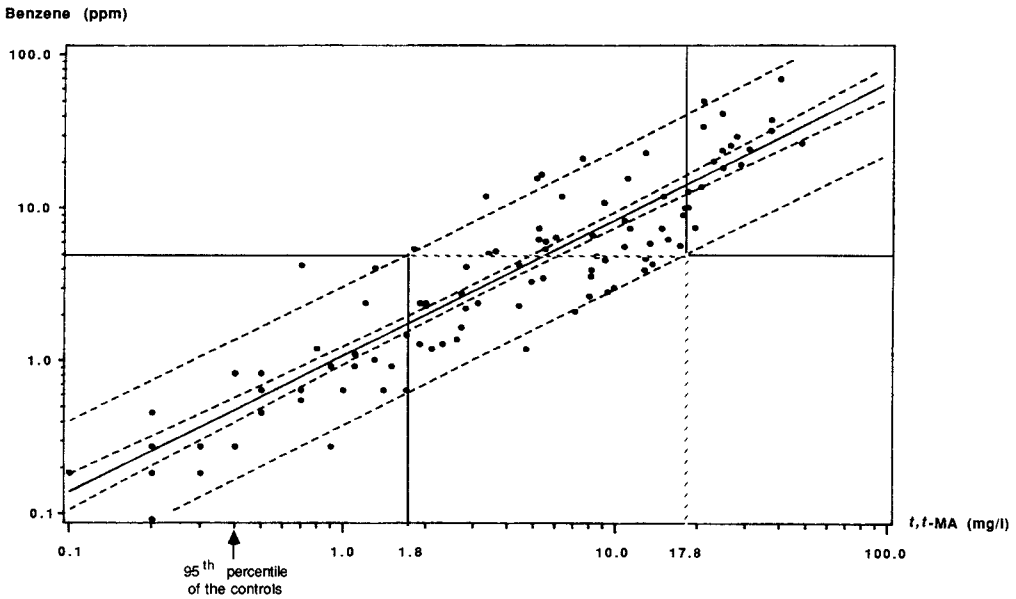

Fig. 1. Relationship between *trans, trans*-muconic acid (t,t-MA) (uncorrected) and TWA benzene concentrations: regression line and 90% confidence interval. t, t-MA: independent variable; benzene: dependent variable

if the exposure was partly percutaneous. Conversely, but from approximately 0.4 mg/l of *t,t*-MA and above, it is possible to estimate the lowest exposure to which a worker has been exposed with a given probability. However, according to our data, this level may be excreted by unexposed people with a probability of 5%. These deductions might be used to establish biological exposure limits taking into account the variability of urinary concentrations after exposure to a given level of benzene and related to the type of investigation being performed. This methodology has been particularly well discussed elsewhere [5].

BEI suggestions

According to our findings, phenol might be easily replaced by *t,t*-MA as a biological indicator with a BEI, as defined by ACGIH, which is obviously dependent on the atmospheric benzene exposure chosen as a threshold. At 10 ppm (as yet the current ACGIH-TLV,, but with a proposed change for that value listed in the Notice of Intended Change), the corresponding BEI should be 10 mg/l (uncorrected). At 5 ppm (current French VME) or 1 ppm (current OSHA-PEL), BEIs could be set at 5 mg/l or 1 mg/l respectively, this last value being largely above the most likely concentration of the controls (0.08 mg/l).

According to the German DFG position on carcinogenic substances, which is not to consider biological tolerance values (BAT) for carcinogenic substances but rather to determine exposure equivalents (EKA), the correlation found between benzene and *t,t*-MA (Table 3) could be used: it is convenient to notice that, after exposure to a given value of benzene expressed in ppm, the corresponding urinary concentration of *t,t*-MA will be approximately the same expressed in mg/l.

Although most of the results of this study and particularly the last one are in accordance with those of Inoue et al. [12], our conclusions about the possibility of separating exposed from unexposed workers are different and in agreement with those of Bechtold et al. [2]. These findings can be explained by better quantifications at low levels, allowed by the easy clean-up procedure used in this study and by the method of using logarithmic transformation before calculating the regression line and its confidence limits.

The various conditions of exposure to benzene observed in this study are considered to be probably representative of other chemical industries which use benzene; nevertheless, we plan to check the agreement of further results with this study, and, particularly, to collect more information about possible biases induced by various coexposures.

Although *t,t*-MA is not the only potential indicator of low benzene exposure, the easy processing of the analytical method together with the high chemical stability of this metabolite, which, furthermore, requires neither hydrolysis nor derivatization, makes it a very convenient substitute for phenol to assess benzene exposures down to around the ppm level on an individual scale.

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