Bull. Environ. Contam. Toxicol. (1999) 63:1-8

Bulletin of Environmental Contamination and Toxicology

Validation of Hippuric Acid as a Biomarker of Toluene Exposure

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Received: 26 January 1999/Accepted: 4 May 1999

Toluene is a commonly used solvent in the rubber, plastics, leather, paint, printing and chemical industries (Nise 1992). Thinner, containing toluene as the major component, is the most used mixture in furniture painting and vanishing in Turkey. Toluene is also an ingredient of shoe adhesives (Burgaz et al 1997). Production of toluene and thinner in Turkey has been raised from 27000 to 39980 tons and from 4258 to 11287 tons respectively during the last three years (DPT 1998).

Since the wide use of toluene numbers of studies has been carried out on occupational toluene exposure. The majority of these studies have focused on describing a urinary metabolite of toluene that is valid to indicate toluene exposure. Hippuric Acid (HA) has been one of the most studied metabolites among the others in order to indicate toluene exposure (De Rosa et al 1985). However, in some recently published studies there have been some doubts on the validity of urinary hippuric acid as an indicator of toluene exposure (Angerer 1985). The presence of hippuric acid endogenously in all individuals and the great interindividual differences in hippuric acid excretion influenced by numbers of factors (diet, medical treatment, alcohol consumption etc.), appear as the major negativity against the reliability of hippuric acid as an indicator of toluene exposure (Inoue et al 1994). Some authors have mentioned that the endogenous presence of hippuric acid in urine might cause some evaluation errors especially in low exposures to toluene (Amorim and Alvarez 1997). Therefore o-cresol has been reported as a more sensitive metabolite in indicating toluene exposure (Angerer and Kramer 1997). In recent studies determination of toluene itself in blood or in urine has been suggested as the most reliable indicator of toluene exposure (Pierce et al 1998).

In spite of the negative interpretations on the validity of hippuric acid in recent publications, numbers of studies have been published proving the relation between hippuric acid excretion and inhaled toluene concentration (Hasegawa et al 1983; De Rosa et al 1987). In this study we aimed to investigate the relation between hippuric acid excretion and toluene exposure especially in low concentrations.

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MATERIALS AND METHODS

Hippuric acid was determined in urine by using Ogata's (1987) method with slight modifications. The chromatography system consisted of a HPLC pump (Jasco, Model PU-980 Intelligent HPLC pump), a UV/VIS detector (Jasco, Model UV-970/975, Intelligent UV/VIS detector) and a LiChrosorb RP18-5, 200 x 4.6 mm column (HICHROM). HPLC mobile phase consisted of a mixture of 5mM KH_2PO_4 (pH: 2.5) / CH_3CN (90/10). A variable flow was used throughout the monitoring of the effluent. The intelligent HPLC pump provided us a programmable flow variation as could be seen in Figure 1. The effluent was monitored at 225 nm and the total assay was carried out at the ambient air temperature.

In order to determine hippuric acid in urine, 1 ml of methanol was added to 1 ml of urine specimen and centrifuged at 2500 rpm for 5 min. Finally 3 µl of the supernatant were injected into the HPLC.

The recovery and reproducibility studies were also performed for known concentrations as described for hippuric acid determination in urine (Table 1).

The survey was carried out with two toluene-exposed groups of workers. Furniture plant: 7 furniture painters were selected for this study. The workers were exposed to thinner containing toluene during the workshift. The painting section consisted of two different small working areas. The distribution of the selected workers among these areas can be seen in Table 2. Shoe plant: 11 workers were selected for this study. The selected workers were exposed to adhesives containing toluene during the workshift. There were also four different small working areas where the employees continuously used adhesive during the workshift. The distribution of the selected workers among these areas can be also seen in Table 2.

Toluene concentration in breathing zone (the air that would most likely be inhaled by the employee) was continuously monitored during the workshift (8 h) by using Miran IBX portable ambient air analyzer in order to calculate the time weighted average (TWA) concentrations of toluene. The workers who worked together in the same small working areas were exposed to statistically the same toluene concentrations because of being very near to each other and doing continuously the same work at the same time intervals. Each of the selected 6 small working areas have the same characteristics. Therefore we decided to use the breathing zone air collection technique to calculate the TWA concentration of toluene.

Eighteen urine samples were collected from the workers occupationally exposed to toluene at the end of the workshift and ten urine samples were sampled among the non-exposed persons as the control group. The

collected urine samples were stored at -25°C until analyzed. Both the control and the exposed groups were asked to complete our previously prepared questionnaires including such items as their smoking habit, alcohol consumption and medical history. The control group was similar to the exposed group with regard to age, alcohol consumption and smoking habits. Both exposed and control groups consisted of persons who were moderate smokers and moderate alcohol consumers according to the questionnaires. Great care has been taken in order to select the nonexposed and the exposed subjects. The smoking and drinking (alcohol) habits of the subjects are almost the same as could be seen in Table 1. Therefore the present study was carried out with limited number of subjects.

Since spot urine samples were used in this study the problem of concentration - dilution effects of urine might cause evaluation errors (Trevisan 1990). Trevisan (1990) reported that concentration in a single urine sample, without adjustment for concentration - dilution effects, is unacceptable because of the wide interindividual variation in urine output. Therefore all of the urinary values were adjusted to creatinine which was measured according to the Baselt (1980) method. The values of control and exposed workers were compared by Students t-test and a level of $p<0.05$ was accepted as significant. Regression analysis was performed to evaluate the correlation.

RESULTS AND DISCUSSION

Urinary hippuric acid was determined using Ogata's (1980) HPLC method. However a slight modification has been made in order to solve the resolution problem and to reduce the total assay time. A variable flow throughout the monitoring of the effluent was used in this study. This modification provided us an appropriate resolution and a reduced total assay time that takes about 8 minutes. The resolution problem, the variation of the flow throughout the monitoring of the effluent and the appropriate resolution after variable flow has been used can be seen in Figure 1. An appropriate recovery and reproducibility were observed for urinary hippuric acid by using the above-mentioned method. The results of the recovery and reproducibility studies have been summarized in Table 1.

This study was performed in collaboration with the National Institute of Occupational Safety and Health and they measured the breathing zone concentrations of toluene in the selected working areas. The National Institute makes also routine health inspections in plants where toluene is continuously used. The small working areas, which were selected for the present study, were also inspected earlier many times and each time the toluene concentrations were measured both by using breathing zone technique and by personal sampling technique in order to calculate TWA concentrations of toluene. According to that earlier routine measurements

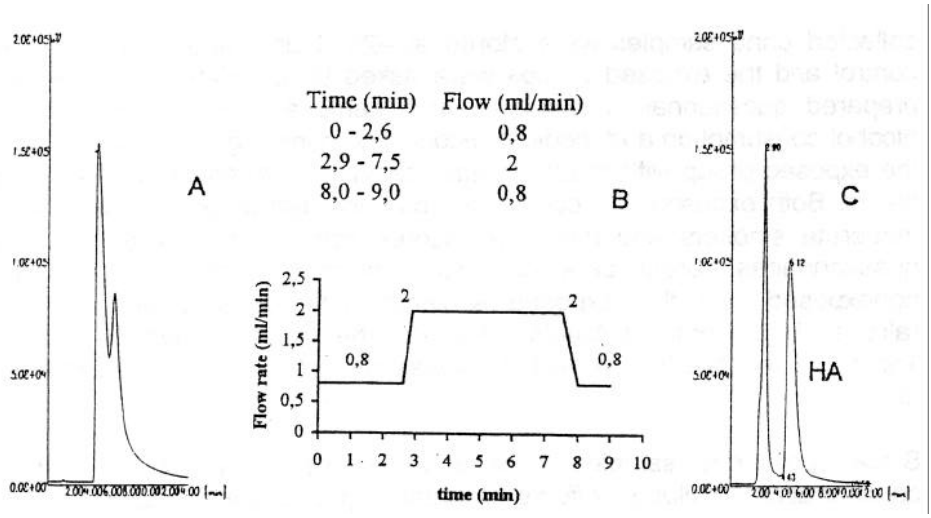

Figure 1. A: HPLC chromatogram of hippuric acid (HA) added control urine before the adjustment of flow variation (The resolution problem can be seen easily). B: The variation of the flow throughout the monitoring of the effluent. C: Hippuric acid added control urine. The difference between the chromatograms in chart A and chart C shows the advantage of using the variable flow in this study.

there was not a statistically significant difference between the TWA concentrations of toluene measured by breathing zone technique or by personal sampling method. Statistically the same TWA concentrations of toluene obtained by the two different air sampling method could be evaluated as a result of the very similar working conditions of the workers and statistically the same breathing zone toluene concentrations in the working area. Accordingly the TWA concentrations of toluene in the working areas were measured by using the breathing zone technique. Therefore the WA concentrations measured in 6 different working areas represent the workers working in the same working area (Table 2).

Table 1. Recovery and reproducibility studies.

Recovery Studies				
	Mean* ± SD (%)	Range (%)		
Hippuric acid	101.9 ± 1.9	98.7 – 103.9		
Reproducibility Studies				
	Actual (mg/ml)	Found** (mg/ml)	SD	CV
Hippuric acid	0.25	0.26 (0.24 – 0.28)	0.02	7.7
	1.00	1.07 (1.02 – 1.10)	0.03	2.8

* The result is the mean of five determinations (1 mg/ml)
** The concentrations are the mean of five determinations.

As shown in Table 2 very low TWA concentrations of toluene were measured in both shoe plant and furniture plant. When we compared the workers for corresponding levels of toluene (TWA) concentrations, the mean urinary excretion of hippuric acid was not significantly different between the workers working in the area of number 1, 2 and 3 ($p>0.05$). Moreover a statistically significant difference could not be observed between the control group and the workers mentioned above ($p>0.05$) with regard to urinary excretion of hippuric acid. In other words a statistically significant dose dependent increment could not be observed between toluene concentrations and the mean hippuric acid excretions in workers working in the area of number 1, 2 and 3 (Table 2).

On the other hand mean urinary concentrations of hippuric acid in the workers working in the area of number 4, 5 and 6 were significantly higher than the control group ($p<0.05$). Moreover a dose dependent relationship was observed between the urinary mean hippuric acid concentrations and the measured TWA concentrations of toluene. The statistically significant relationship between the mean hippuric acid excretions and the toluene concentrations in workers working in the area of number 4, 5 and 6 could be seen in Figure 2.

The results of this study prove the earlier results that urinary hippuric acid excretion is not valid to indicate low exposure of toluene. However a statistically significant increase of urinary hippuric acid was observed in the workers exposed to toluene of 44, 66 and 115.2 ppm (Figure 2). In order to calculate the equation ($y=0.0166x+0.0404$) of the relationship, the mean hippuric acid and the toluene concentrations observed in workers working in the are of 4, 5 and 6 were subjected to the linear regression analysis. The equation consisted of the values that are significantly higher than the control group and the R^2 value of 0.9982 proved the significance of the linear dose dependent increment. However the intercept value of the equation (0.0404) is very near to zero and does not indicate the endogenous hippuric acid excretion. Therefore the equation itself may also prove the insufficiency of urinary hippuric acid to indicate the low exposures to toluene.

When the workers working in the area of number 1, 2 and 3 were taken into account for corresponding levels of toluene, the hippuric acid concentrations were not statistically different from the control group ($p>0.05$). Therefore these mean hippuric acid values, representing the toluene exposure of 12.3, 15.5 and 17.8 ppm, were not included to the first equation mentioned above. The second equation ($y=0.0018x+0.3144$) consisted of the mean hippuric acid concentrations of the workers working in the area of number 1, 2, 3 and of the control group (Figure 2). The R value of 0.3615 proved the lack of the dose dependent increment of hippuric acid excretion in the workers exposed to low levels of toluene.

Table 2. The distribution of the workers among the working areas and the characteristics of the workers.

Parameters	Control	Shoemakers				Furniture makers	
n (Total)	10	11				7	
Ages	29.62± 9.89 (19 - 50)	28 ± 7.5* (17 - 48)				28.42 ± 6.91 (17 - 37)	
Years of employment	-	5.81 ± 5.28 (1 - 18)				2.36 ± 1.36 (1 - 5)	
Alcohol consumption	Moderate †	Moderate				Moderate	
Smoking habit	Moderate ‡	Moderate				Moderate	
Working areas	-	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6
n	10	3	2	3	3	2	5
Toluene (ppm)**	-	12.3[a]	15.5[a]	17.8[a]	115.2[b]	44[b]	66[b]
Hippuric Acid, g/g creatinine	0.31±0.06 (0.25-0.46)	0.37±0.01[a] (0.35-0.38)	0.32±0.08[a] (0.26-0.38)	0.35±0.17[a] (0.23-0.55)	1.97±0.76[b] (1.14-2.64)	0.79±0.09[b] (0.72-0.86)	1.11±0.23[b] (0.82-1.44)

* Mean ± St. Dev. (Range)
** TWA (Breathing zone)
† 1 glass of Raki (20% of alcohol) per day
‡ 5 - 10 cigarettes per day
[a] Hippuric acid excretion was not significantly different from the control group ($p>0.05$)
[b] Hippuric acid excretion was significantly different from the control group ($p<0.05$)

The last assumption was also confirmed by the slope value (0.0018) of the equation.

We obtained two lines using the two equations as shown in Figure 2. The interception point of the two lines might be evaluated as the minimum toluene concentration that causes an elevation of the urinary hippuric acid excretion. The interception point of the two lines corresponded to toluene concentration of 18.51 ppm and to hippuric acid concentration of 0.35 g/g creatinine (Figure 2). Accordingly, exposure to toluene higher than 18.51 ppm might causes an elevation of hippuric acid excretion.

In Turkey, 200 ppm is the maximum allowable concentration for toluene in the workplaces. According to our results urinary hippuric acid is capable to indicate the toluene exposure of higher than 200 ppm and even at concentrations under the maximum allowable concentration. Therefore the urinary hippuric acid could be still suggested as a good indicator of toluene exposure on condition that the toluene exposure should be higher than 18.51 ppm according to our results. The lack of correlation between hippuric acid excretion and toluene exposure in workers working in the area of number 1, 2 and 3 is apparently due to the rather high background level of hippuric acid.

The urinary hippuric acid levels in the control group were observed relatively lower than the earlier studies (De Rosa et al 1985; Nise 1992).

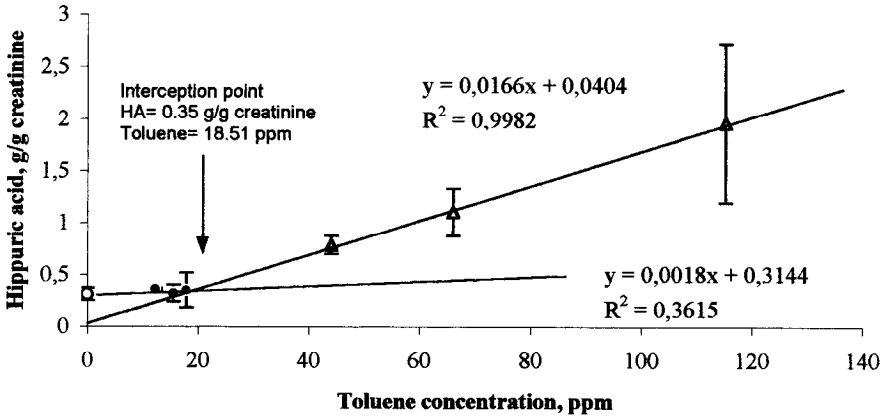

Figure 2. The relationship between hippuric acid excretion and toluene exposure.

o: Mean hippuric acid excretion of the control group.

•: mean hippuric acid excretion of the workers working in the area of number 1, 2 and 3

∇: Mean hippuric acid excretion of the workers working in the area of number 5, 6 and 4.

However Nise (1992) described a significant relation between alcohol consumption and reduced hippuric acid concentration in urine. The relatively low hippuric acid excretions observed in the control group might be explained by the alcohol consumption of the subjects.

o-Cresol is the other urinary metabolite of toluene and has been suggested as a more specific indicator of toluene exposure (Angerer and Kramer 1997). However only a small proportion of the retained toluene is excreted as *o*-cresol (<1%) (Nise 1992). Therefore the validity of this metabolite for biological monitoring in low exposures to toluene is also questionable (De Rosa 1987; Nise 1992).

Consequently many studies including our study have suggested hippuric acid as a valid indicator of toluene exposure especially in high levels. Moreover hippuric acid is still in use in many countries as an indicator of toluene exposure. The simplicity of the determination methods of urinary hippuric acid has also enhanced the importance of this metabolite for the underdeveloped and developing countries. The modified determination method used in this study could be also useful for poorly equipped laboratories because of the minimum equipment requirements.

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