Hippuric acid in urine: reference values
Ácido hipúrico em urina: valores de referência

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Keywords

Abstract
Objective
To establish reference values for hippuric acid (HA) excreted in the urine, and to evaluate the impact of age, gender, alcohol, and tobacco, on these levels in a population nonexposed to toluene.

Methods
Reference values for hippuric acid in urine were determined in 115 toluene nonexposed healthy volunteers, from Alfenas city, Southeastern Brazil. A questionnaire was applied to each volunteer and data on occupational and personal habits were collected. Biochemical and hematological analyses were used to confirm the volunteers’ good health condition. Reference values were expressed in g HA/g urine creatinine, as mean ± standard deviation (x ± SD), median, 95% confidence interval (95%CI), 95th percentile, and upper reference value (URV, mean +2 SD).

Results
Reference values of hippuric acid in urine were: mean ± standard deviation =0.18±0.10; median =0.15; 95% confidence interval =0.16±0.20; 95th percentile = 0.36 and upper reference value (URV, mean +2 SD) =0.38. Statistically significant differences in urinary HA (Wilcoxon – Mann/Whitney, $p < 0.05$) were observed for different genders and age groups. Alcohol ingestion and smoking habit did not significantly affect the results.

Conclusions
The reference values of hippuric acid in urine can be used in biomonitoring programs of workers occupationally exposed to toluene, especially in the southern region of the state of Minas Gerais. Age and gender may affect the HA reference values.

Descritores

Resumo
Objetivo
Estabelecer valores de referência para o ácido hipúrico (AH) em urina e avaliar a influência da idade, sexo, uso de bebidas alcoólicas e de tabaco nestes níveis, numa população não exposta ao tolueno.

Métodos
Os valores de referência para o ácido hipúrico em urina foram determinados numa população de 115 voluntários da cidade de Alfenas, MG, saudáveis e não expostos ocupacionalmente ao tolueno. Informações sobre hábitos no trabalho e pessoais,
INTRODUCTION

Toluene is the most popular solvent used in industry and it can occur ubiquitously as an environmental contaminant to which the general population is exposed.23,25 Gasoline, paints, strippers, glues and some household cleaners, and tobacco smoke may be sources of air toluene.20

Toluene is primarily metabolized to benzoic acid that conjugates with glycine to yield hippuric acid, excreted in urine. Hippuric acid is still the most used indicator in biomonitoring of toluene exposed workers for it shows a good correlation with the exposure level despite its lack of specificity to toluene or occupational exposure.5

The most important sources of background hippuric acid are environmental toluene contamination and dietary food, such as fruits (plum and peach), coffee green beans and other benzoic acid liberators.8,16,24

In the last years the air concentration of toluene in occupational environment is decreasing mainly due to better hygiene conditions, and the levels of bioindicators are coming closer to their background levels. Thus, for properly interpreting biomonitoring results, it is necessary to establish reference values (RV) in a non-occupationally exposed population.1,2,6

The purpose of the study was to determine background levels of HA excretion in the urine, and to evaluate the influence of alcohol, tobacco, age and sex on these levels in a toluene occupationally nonexposed population to.

METHODS

Reference population and exclusion criteria

The volunteers of the reference population were randomly selected among residents of the city of Alfenas, an urban area located in the south region of the state of Minas Gerais, Brazil, taking into account their age and gender, as well their consumption of tobacco and alcohol for it is recommended 50 subjects for each subgroup studied.7 This is an important agricultural area with low traffic density and low industrialization. A total of 142 subjects (61 men and 81 women) with no occupational exposure to toluene, aged 18-60 years (mean ± SD =33.5 ± 9.6) were asked to answer a toxicological protocol at the time the samples were taken, giving information related to their personal habits (smoking, alcohol intake, leisure activities), residence, age, work, types of food consumed, drug ingestion, among others. Healthy subjects are required in studies for determining reference values of bioindicators.2,14 Beside questionnaire data, health status was assessed by laboratory determination of some biochemical and hematological parameters: blood glucosis, total cholesterol, transaminases (AST e ALT), urea, triglycerides, total proteins, serum and urine creatinine, urine analysis and hemogram.

Unless exposure to toluene had been stated as the main exclusion criteria, other factors were taken into account for exclusion: any pulmonary, hepatic, and renal illness or complaints of any sort about their health (7 subjects); abnormalities of any biochemical or hematological parameters (15 subjects); sub-
jects who ingested preserved food containing benzoic or benzoate within the previous 24 hours (3 subjects); and those chronically taking any medicines (2 subjects). The research protocol was approved by the University’s Ethical Committee on Research.

**Biological samples**

Fasting blood samples (12 ml) were drawn using a vacuum collection system with heparin and biochemical and hematological analysis performed on the same day. Spot urine samples (50 ml) were collected in polyethylene bottles and creatinine was tested before acidification and frozen at -20ºC until analysis (at a maximum of 4 days after sampling).

**Hippuric acid analysis**

Solutions of hippuric acid (Sigma) were prepared at concentrations of 10 and 5 g/L. An internal standard solution, 0.4 g/l heptadecanoic acid (Sigma), was prepared in methanol. HA extraction from urine was performed according to Kira et al.17 (1977) method, and identified using the Alvarez-Leite et al.3 (1994) gas chromatography technique. Calibration curves were prepared adding scaled quantities of hippuric acid in urine to obtain 0.1, 0.2, 0.5, 0.8, 1.0, 1.2 and 1.5 g/L concentrations (r = 0.9934). Three calibrators were analysed daily (0.1, 0.5 and 1.0 g/l) along with the assay of samples to be evaluated. The quantification limit (LOQ) for HA in urine was 0.05 g/L. The assay precision was determined by analyzing samples containing 0.2, 1.0 and 1.5 g HA/L. The intra-assay variation coefficient was 8.2% (10 samples for each concentration) and inter-assay variation was 10.2% (for samples analyzed daily during 10 days).

The results were expressed in g HA/g urine creatinine (Merck kit).

**Statistical analysis**

Due to non-normal data distribution, nonparametric Wilcoxon Man-Whitney Z test was used to compare urinary HA concentration between subgroups (Statgraphics, Statistical Graphics System, STSG). Differences were considered statistically significant at p<0.05.

**RESULTS**

According to the study exclusion criteria, 27 volunteers (19%) were excluded and data from 115 occupationally nonexposed subjects were evaluated. The median hippuric acid was 0.15 g/g creatinine, 95th percentile of 0.36 g/g creatinine (Table 1).

Figure shows the reference values distribution in

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<tr>
<th>Table 1 - Reference values of urinary hippuric acid (g/g creatinine) in occupationally nonexposed subjects and in the population subdivided according to the gender and age groups.</th>
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<tbody>
<tr>
<td>Hippuric acid (g/g creatinine)</td>
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<td>N</td>
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<td>Mean ± SD</td>
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<td>Median</td>
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<td>95% Confidence range</td>
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<td>Upper reference value (mean +2 SD)</td>
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<td>95th Percentile</td>
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*male/females Z=2; P<0.05; **18-35 / 36-60 years Z= 2.07; P<0.05.

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<th>Table 2 - Hippuric acid in urine (g/g creatinine) evaluated in the total general population and according to the smoking and alcohol intake habits.</th>
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the general population. Urinary HA levels were significantly different between males and females and in age groups (Table 1).

Alcohol ingestion or tobacco smoking did not significantly change the urine basal levels of hippuric acid in the general population (Table 2).

**DISCUSSION**

The availability of a well assessed reference values is fundamental for biomonitoring subjects nowadays occupationally exposed to toluene since current levels of exposure are gradually decreasing.

The study was carried out in the urban area of the city of Alfenas that presents characteristics of traffic and industrialization (at a low level) very similar to other cities of the southern region of Minas Gerais. Thus, the reference values established can be regarded as valid for this region. The population size was estimated according to the criteria recommended by the International Federation of Clinical Chemistry to establish reference values for clinical laboratory parameters.

The population was randomly selected, regardless the occupational activities, and met the recommended characteristics necessary for establishing reference values, i.e., healthy, occupationally nonexposed, age ranging from 18 to 60 years old, both sexes with similar dietary habits.

In the 115 volunteers occupationally nonexposed to toluene, mean urinary hippuric acid was 0.18 g/g creatinine (corresponding to a mean of 0.28 g/L). This result was lower than the mean reference value reported by Alvarez-Leite et al., 1998, for the metropolitan region of Belo Horizonte (0.42 g/L). This is probably due to the different environmental chemicals that pollute these regions. Buchet & Lauwerys, 1973 reported a mean level of urinary hippuric acid of 0.80 g/g creatinine in a control group occupationally nonexposed to toluene. Villanueva et al., 1994 reported a geometric mean of 0.09 g/g creatinine and 0.11 g/g creatinine in Japanese (n=43 males) and Philippine (n=34 males) people as HA background values. However, these authors’ studies were not aimed to define hippuric acid reference values.

As reference values are intended to characterize the upper margin of the current background exposure of the general population to a given chemical, it seems more reasonable to use the upper reference value or the 95th percentile (in a non-normal distribution) to evaluate individual data of exposed workers while distinguishing occupational and non-occupational exposure. When evaluating the toluene exposed workers in a group basis one has first to assess the comparison with the distribution of reference values.

In Brazil, 1994, the adopted cut-off value of urinary hippuric acid was 2.5 g/g creatinine (IBMP) in the exposed population. The reference values found were low, and this fact reinforces the use of urinary hippuric acid as a bioindicator of toluene exposure, despite its lack of specificity.

For evaluating reference values there must be considered a variety of factors that can affect these values, such as age, gender, consumption of alcohol and tobacco, drug ingestion, leisure activities, living and working places, among other, that can change the basal bioindicator levels. It is known that preparations containing benzoic acid can rise the HA excretion. All these data were obtained from each volunteer through a questionnaire and they were used to subject exclusion as well as to evaluate the results.

No significant differences in HA excretion between subgroups of smokers/non-smokers and alcohol drinkers and non-drinkers were observed. There were classified as smokers people smoking more than 5 cigarettes a day and as drinkers those consuming any alcohol beverage at least 4 times a week (a minimum of 20 g alcohol a week).

There are conflicting reports about the influence of alcohol and tobacco in the metabolism of toluene affecting its metabolites elimination, mainly under experimental and occupational exposure circumstances. It was found only one paper reporting that the individual’s smoking and drinking habits, either one of them or both, did not significantly change basal HA levels.

Gender seems to affect HA background levels: females show higher values than males. Hassegawa et al., 1983 reported similar observations in workers occupationally exposed to toluene. In the two age groups studied there was statistical difference (p<0.05) in HA excretion, which was higher in the older group. There were no references in the literature concerning this aspect and further studies are needed to clarify the importance of age in determining basal HA levels.
REFERENCES


