Introduction

Among the aromatics hydrocarbons group toluene is an organic solvent widely used on different industrial processes such as pints, shoes and coke production along with print processes among others. Besides, people can be exposed by breathing automobile gas exhaust, working with gasoline or combustible oils and paints and even though its direct inhalation [1-6]. People exposed chronically to toluene, both occupationally and through recreational glue sniffing, have demonstrated anatomical and irreversible changes in the brain, as well as neurobehavioral impairment reported as neurological damage or chronic toxic encephalopathy [5,6]. Recently, it has been reported that toluene present genotoxic effect on human population associated with genetic susceptibility [7,8].

The main route of exposure is through inhalation of its vapours [2,5]. A fraction of the absorbed toluene is excreted without changes in the expired air. The major part of this solvent is biotransformed in liver to benzoic acid which is then conjugated with glycine to hippuric acid [1,2,9-12]. Less than 1% of the toluene absorbed is metabolised to o-cresol which is transformed to sulphate or glucuronic derivatives. Among them, the Urinary hippuric acid has been intensely used as occupational biomarker to toluene. This compound present a good correlation with the degree of exposure being one of the recommended biomarkers to assess exposure to this solvent [1,2,13]. Nevertheless, the use of only this biomarker could lead to overestimate the exposure due to hippuric acid is commonly found in urine samples of people which consume food containing benzoates as additives [2,3,11,12,14]. There are reports that show the level of hippuric acid of occupationally non-exposed population even overcoming the biological exposure index (BEI) probably due to this fact [3,12].

Recently Bahrami et al. reported that hippuric acid take relevance as indicator when the environmental exposure is near the Threshold Limit Value (TLV) of 50 ppm [15]. Despite of its low production, o-cresol is not a common constituent of normal urine and its determination is a more reliable and sensitive tool for monitoring toluene exposure. This author reported a good correlation with toluene in ambient ten folds lower the TLV, showing its utility at low exposure level [11,15]. Currently there are different assays for the quantification of the toluene related biomarkers, including liquid and gas chromatography, coupled to ultraviolet, mass and flame ionization detectors methods [3,4,11,12,15-20].

The aim of this work was to develop and optimize a simple method for the determination of urinary o-cresol by gas chromatography-flame ionization. The application of the method in real samples is presented.
Experimental

Chemicals

O-Cresol P.A and Nitrobenzene P.A were purchased from Fluka. Sodium Chloride P.A., Anhydride Sodium Sulphate P.A. and Hydrochloric Acid P.A. were purchased from Merck Quimica Argentina (Buenos Aires, Argentine). Diethyl Ether Analytical Degree and Methanol Analytical Degree were from Aberkon Quimica Argentina (Buenos Aires, Argentine). Double distilled water (pH 5) was obtained with a Figmay distillatory system (Córdoba, Argentina).

Chromatography

The chromatographic procedure involved a Hewlett Packard 5890 gas chromatograph coupled to a Flame ionization detector and an auto sampler model Hewlett Packard 6890. A column (1%-phenyl) methylpolysiloxane (HP-1, 30 m x 0.25 mm, 0.25 um film thickness, J&W Scientific, Folsom, CA) was used. The temperature program was performed as follows: initial temperature: 70ºC for 11.00 min then a ramp of 20 ºC/min to 160ºC and a final step of 5 min at this temperature. The injector port and detector were set at 250 and 275ºC respectively. The carrier gas used was nitrogen at 1.2 mL/min. The injected volume was 2µL in split mode (1/10).

Standard solution and urine samples

An o-cresol stock solution was prepared in methanol to a final concentration of 2 mg/mL. A working standard solution with a final concentration of 20 µg/mL in methanol was prepared from the stock solution.

A Nitrobenzene stock solution was prepared in methanol to a final concentration of 10 mg/mL. A working standard solution with a final concentration of 250 µg/mL in methanol was prepared from the stock solution.

Blank human urine, was spiked with o-cresol to reach a final concentrations of 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 and 3.0 µg/mL. Urine samples were collected from young healthy people none exposed to toluene.

All solutions and urine samples were stored at 4ºC until analysis.

Studied population

All the analyzed samples reported in this work came from people with clinical indication for determining urine o-cresol as indicator of toluene exposure and decided carry out the analysis at the Analytical Poisoning Control Center (CENATOXA).

Sample preparation and extraction procedure

Aliquots (5 mL) of blank and spiked urine samples were loaded onto capped tubes. One mL of concentrated hydrochloric acid was added to all tubes and incubated for 90 minutes at 95ºC in the hydrolysis step. After incubation, tubes were let to cool at room temperature. Nitrobenzene working solution (100 µL) was added as internal standard and mixed vigorously with a vortex. A salting out was performed adding 1g of solid sodium chloride. Two mL of diethyl ether was added as extraction solvent and mixed in vortex for two minutes. The organic layer was placed into clean vials containing anhydride sodium sulphate to eliminate the possible humid present.

Once the extracts were dehydrated they were placed into vials for injection.

Method Validation

Recovery, precision and linearity

The absolute recovery of o-cresol was assessed on spiked urine specimens at three concentration levels (0.2, 0.5 and 3.0 µg/mL). The extraction efficiency was determined by comparing the detector responses obtained by the analysis of fortified samples with the resulting from direct injections of equivalent quantities of standard solutions. Within-day precision involved four measurements of the same samples within a single run. Day-to-day precision involved the estimation of the run to run variation of the same samples for three consecutive days.

Linearity of standard curves

Three sets of four 5.0 mL urine samples spiked with o-cresol working solution resulting in a final concentration of 0.2, 0.5, 1.0 and 3.0 µg/mL and nitrobenzene working solution resulting in a final concentration of 5 µg/mL were used.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Both the LOD, defined as the lowest concentration that the analytical process can reliably differentiate from background levels, and the LOQ, defined as the lowest concentration that can be measured with stated level of confidence, were calculated as described by Quattrochi and Shah [21,22]. Briefly, LOD was estimated by the injection of five blank urine samples and the integration of baseline noise of the system in the area covering the mean retention time of o-cresol was assessed. The LOD was calculated as three times the mean baseline noise [21]. For LOQ estimation, four replicate at three concentrations (0.05, 0.1 and 0.2 µg/mL) were tested. The LOQ was estimated as the lowest concentration which showed a coefficient of variation (CV %) less than 20% [22].

Application of the assay to the biological monitoring. Seventy eight urine samples were analyzed using the described methodology. People were interviewed about social habits (alcohol and tobacco consumption), dietary and consume of medicaments along with occupational history. All the solutions and urine samples were stored at 4ºC until assay.

Results and Discussion

In a first attempt to extract o-cresol from urine, the extraction was performed according to the procedure described for the determination of phenol and para-cresol (p-cresol) in urine [23]. The use of diethyl ether as solvent of extraction resulted easier to handle compared to the use of dichloromethane as described by Truchon et al. [11]. We found a better layer separation improving the process by adding a salting out step instead of the cooling treatment proposed.

Carrier gas flow and thermal condition for inlet, oven and detector were also tested. The best chromatographic conditions of sensitivity and resolution found are described above. The oven temperature ramp was enough to avoid the matrix interferences respect to the metabolite to investigate, especially when it was observed a non constant peak in some blank urine samples (Figure 1). Apart from

Citation: Navoni J, Riddoff A, Olivera M, Alvarez G and Lepori EV. Quantitative Analysis of Urinary O-Cresol by Gas Chromatography - Flame Ionization Detection for the Monitoring of Population Exposed to Toluene. SM Anal Bioanal Technique. 2018; 3(2): 1018.
that, the possibility of overlapping of o-cresol and nitrobenzene with other substances such as phenol, p-cresol and Meta cresol (m-cresol) were also tested. No interferences were observed. Every compound presented enough difference in retention times at the proposed chromatographic conditions to neglect them.

The mean recovery rate, within-day and day-to-day coefficients of variation were assessed at three different concentration levels (0.2, 0.5 and 3.0 μg/mL) Table 1. The procedure described presented an extraction efficiency rounding 100%. The use of internal standard avoided possible variation especially in the extraction and injection steps.

The chromatographic conditions resulted in an optimal run length of 20.50 minutes. The mean retention time for o-cresol was 8.2 minutes and for nitrobenzene was 8.6 minutes.

The concentration range for the calibration curve was selected according to the Biological Exposure Index (BEI) recommended by ACGIH and SRT [13,24]. The linearity of the method was tested in the range of concentration selected. The linear detector response was observed along the tested range of concentration Table 2. The results of LOD and LOQ established as described above were 0.03 μg/mL, and 0.20 μg/mL respectively.

In Table 3 is shown the descriptive evaluation of the results obtained on the total of real urine samples analyzed. The results were divided in two groups taking into account the information obtained in the interview. Seventy nine percent of the samples were from the group SM.

Table 1: O-Cresol recovery, within-day and day-to-day precision at three concentrations.

| Concentration (μg/mL) | Recovery (%) | Intra-day precision (%CV) | Inter-day precision (%CV) |
|-----------------------|--------------|---------------------------|---------------------------|
| 0.2                   | 98.3         | 10.0                      | 12.5                      |
| 0.5                   | 110.6        | 5.3                       | 10.0                      |
| 3.0                   | 95.4         | 2.3                       | 6.8                       |

CV: Coefficient of Variation

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Table 2: Analysis of calibration curve parameters.

| Range of concentration (µg/mL) | 0.20 - 3.00 |
|-------------------------------|-------------|
| Coefficient of correlation ± SD | 0.995 ± 0.003 |
| Slope ± SD                   | 0.2822 ± 0.02 |
| Axis intersection ± SD        | 0.0221 ± 0.002 |
| Points of calibration         | 4           |

Data obtained from five curves prepared and processed independently.

- Results obtained expressed as mean ± standard deviation.

Table 3: Urinary o-cresol concentration in exposed and non-exposed population.

| Group | Non exposed | Exposed |
|-------|-------------|---------|
| Number | 62          | 16      |
| Positive samples a | 8           | 15      |
| Negative samples b | 54          | 1       |
| Mean a | 0.45        |         |
| Median a  | 0.36        |         |
| Minimum  | ND          |         |
| Maximum c | 0.81        |         |

a- Samples with chromatographic signal at o-cresol retention time.
b- Samples with no chromatographic signal at o-cresol retention time.
c- Statistic calculated with quantifiable values.

- All results are expressed in µg/mL.

The developed method was successfully applied to urinary o-cresol quantification in real samples from exposed and non-exposed population demonstrating its usefulness for assessing toluene exposure.

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