Determination of 2,5-Hexanedione in Urine by Headspace Solid-Phase Microextraction and Gas Chromatography

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Abstract

2,5-Hexanedione (2,5-HD) is the most important metabolite of n-hexane and methyl ethyl ketone in human urine. Urinary 2,5-HD is used as a biomarker for biological monitoring of workers exposed to n-hexane. A simple method using headspace solid-phase microextraction (HS-SPME) and gas chromatography (GC) equipped with a flame-ionization detector (FID) was developed. The parameters that affect the HS-SPME–GC–FID process were optimized (i.e., fiber coating, sample volume, adsorption and heating time, salt addition, and extraction temperature). The assay presented linearity in the range of 0.075 to 20.0 mg/L, precision (coefficient of variation < 7.0%), and detection limit of 0.025 mg/L for 2,5-HD in urine. The method was successfully applied to the analysis of 2,5-HD in urine samples from eight workers occupationally exposed to n-hexane in shoemaker’s glue.

Introduction

n-Hexane is an important aliphatic compound widely used in industry to make glue, paints, varnishes, and printing inks and in shoe manufacturing and repair. Occupational or experimental exposure to n-hexane can lead to serious impairment of the nervous system, classified as a central-peripheral distal degenerative axonopathy (1).

Toxicological studies have shown that 2,5-hexanedione (2,5-HD), an active metabolite, is the cause of the peripheral neuropathies that manifest themselves as leg weakness and progress to paralysis in chronic n-hexane exposure (2). 2,5-HD probably reacts with neurofilament lysine-amines to yield 2,5 dimethylpyrrole adducts, which are critical to the mechanism of toxicity. Alkylpyrroles are susceptible to antioxidative dimerization with subsequent cross-linking of the neurofilaments (3).

Analysis of 83 different commercial glue brands in Brazil demonstrated that n-hexane was the main solvent present (4). The exposure of workers predominantly occurs in the glue industry and shoe manufacture and repair, mainly by inhalation, whereas dermal absorption occurs only by direct skin–glue contact.

The most widely used indicator in the monitoring of workers exposed to n-hexane is urinary 2,5-HD, and many studies have demonstrated good correlation between exposure to n-hexane in workplaces and 2,5-HD urinary excretion (5–7). Urinary 2,5-HD is recommended as a better approach than air monitoring in the assessment of health risks, specifically for the early detection of n-hexane neurotoxicity (8).

The use of total 2,5-HD (determined after urine hydrolysis) as opposed to free 2,5-HD for biological monitoring of exposure to n-hexane has been the basis of considerable controversy (9–14). The American Conference of Governmental Industrial Hygienists (ACGIH) (15) recommends the determination of free 2,5-HD in urine collected at the end of a working shift at the end of the workweek and a biological exposure index (BEI) of 0.4 mg/L. This decision was made because the other n-hexane metabolites (4,5-dihydroxy-2-hexanone and 5-hydroxy-2-hexanone) are converted into 2,5-HD during hydrolysis. 2,5-HD and free 2,5-HD are both suitable from an analytical point of view and meaningful for biological monitoring purposes because the neurotoxic risk arising from conjugated metabolites is not relevant (12,16,17).

Gas chromatography–mass spectrometry (GC–MS) and GC–flame-ionization detection (FID) are the techniques most widely used for the quantification of 2,5-HD in urine (13,14,18–20), in addition to electron capture detection (ECD) after derivatization (10) and high-performance liquid chromatography using UV, fluorescence detection, or MS (21,22). However, whatever method is used for identification, the analyte should be extracted from the biological matrices. Liquid–liquid and solid-phase extraction are commonly used procedures (9,10,13,14).

Solid-phase microextraction (SPME) is a very sensitive and selective process of extraction that integrates sampling, extraction, concentration, and sample introduction into a single
samples collected from volunteers occupationally exposed to 2,5-HD. The method was validated and applied to the analysis of urine. The SPME extraction were optimized, the most important HD in human urine. Various factors affecting the efficiency of capillary GC–FID is described for the determination of free 2,5-HD.

A factorial experimental design was performed to determine the optimum conditions for the analysis of 2,5-HD in human urine. The variables selected as potentially affecting the extraction efficiency were adsorption time (5–15 min), extraction temperature (40–60°C), and previous heating time (5–15 min). The order of the experiments was fully randomized to provide fair results.

The results of this design allowed us to evaluate the significant variables so that we could plan a subsequent higher order design with central composite, which was performed in the same procedure. The variables evaluated were extraction temperature (46–74°C) and adsorption time (8–22 min). In all cases, data analysis was performed by means of the

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**Experimental**

**Chemicals and preparation of standards**

2,5-HD was purchased from Sigma-Aldrich (St. Louis, MO), and 5-methylhexanone-2 (internal standard, IS) from Merck (Darmstadt, Germany). Stock solutions (50 mg/L of 2,5-HD and 100 mg/L of the IS) were prepared in water purified using a Millipore Milli-Q Plus system (São Paulo, Brazil) and stored at 4°C for one week maximum. Dilute solutions were prepared just before use. Organic solvents and other chemicals and reagents were of analytical grade.

**Equipment**

The following fiber coatings were compared to evaluate their performance in the 2,5-HD extraction: 100-µm polydimethylsiloxane (PDMS), 65-µm carbowax/divinylbenzene (CW/DVB), 75-µm Carboxen/PDMS (CAR/PDMS), and 70-µm PDMS/DVB. These fibers were purchased from Supelco (Bellefonte, PA), and they were conditioned for 2 h in the injection port of the GC at 250°C, 220°C, 300°C, and 250°C, respectively. Magnetic stirrers and manual fiber holder (Supelco), water bath with digital temperature control interface and circulation mechanism for heating (B. Braun Biotech International GmbH, Melsungen, Germany), and PTFE/silicone septum and 15-mL HS flasks (Supelco) were employed. Purified water was obtained by using a Milli-Q Plus system from Millipore.

A GC system CG1000 (Ciola Gregori, São Paulo, Brazil) equipped with splitless injector inlet liner, interfaced to a PC with DANI DS 1000 integrator (Dani Strumentazione Analitica, Monza, Italy), and IQ3 software for data acquisition were used.

**Samples from human volunteers**

The urine samples were obtained from 10 healthy volunteer nonsmokers who had not been exposed to n-hexane or any other solvent to optimize and to validate the method. Samples of occupationally exposed workers were obtained from eight individuals handling leather glues containing n-hexane in the shoe industry. These samples were collected at the end of the shift at the end of the workweek as recommended by the ACGIH (15). Spot urine specimens were collected directly from the donors in 50-mL polyethylene flasks and kept frozen at –20°C until analysis, which was performed up to seven days following collection. The study was approved by the Research Ethics Committee of the Institution and the individuals signed an informed consent form to participate, after receiving information about the aim of the research.

**Method development**

Optimization of chromatographic conditions. A capillary column 100% PDMS ZB-1° (30 m x 0.53-mm i.d., 5.0-µm film thickness) was used. The oven temperature program was initial temperature of 80°C for 6 min, increasing at 15°C/min until 170°C, and holding for 3 min. Injector and FID detector temperatures were 250°C. Carrier gas was nitrogen at a flow-rate of 8.0 mL/min, and IS was 5-methylhexanone-2.

HS-SPME optimization. A first simple factorial plan (23) was carried out to distinguish the significant parameters by analysis of the effects. The variables selected as potentially affecting the extraction efficiency were adsorption time (5–15 min), extraction temperature (40–60°C), and previous heating time (5–15 min). The order of the experiments was fully randomized to provide fair results.

The results of this design allowed us to evaluate the significant variables so that we could plan a subsequent higher order design with central composite, which was performed with the same procedure. The variables evaluated were extraction temperature (46–74°C) and adsorption time (8–22 min). In all cases, data analysis was performed by means of the
statistical package Statistic (V. 5.5, Statsoft, Tulsa, OK). Univariate assays were performed to evaluate other variables such as coating material of the fiber, salt addition, sample volume, time, and temperature of desorption in the GC injection port.

Sample preparation. Two milliliters of human urine and 0.5 mg/L of IS were transferred to the 15-mL HS flask, and 1 g of Na$_2$SO$_4$ and a 1-cm stirring bar were added. The vial was rapidly sealed with a PTFE-septum cap and heated at 50°C in a water bath, under magnetic stirring, for 10 min, and PDMS/DVB fiber was exposed in HS for 20 min to allow adsorption of the compounds. Then, the fiber was retreated from the flask and immediately inserted into de-heated GC injection port at 250°C for 2 min.

Method validation

The method was validated for linearity, precision, detection and quantification limits, and extraction efficiency; these studies were conducted in urine samples from nonsmoking, non-occupationally exposed donors (blank samples). The standard calibration curve was obtained by analysis of the spiked urine with concentrations 0.075, 0.5, 2.0, 5.0, 10.0, and 20.0 mg/L of 2,5-HD and 0.5 mg/L of the IS ($n = 5$ per concentration) and using the procedure outlined previously. Plots of 2,5-HD/IS peak-area ratios versus analyte concentration were constructed and the relationships were determined by linear least-squares regression analysis. The detection (LOD) and quantification limits (LOQ) were determined using the standard deviation of the results obtained after analysis of 10 independent urine sample blanks. The LOD was 3 times the standard deviation of blank samples, and the LOQ was 10 times the standard deviation. The intra-assay precision was estimated by analysis of urine samples spiked with 0.075, 0.5, 2.0, 5.0, 10.0, and 20.0 mg/L of 2,5-HD concentrations ($n = 5$ per concentration). The interassay precision was determined for three consecutive days of urine samples spiked with 0.15, 2.0, and 10.0 mg/L of urine, taking into account the vial capacity (15 mL). Slight differences in absolute peak areas were observed, the best result being obtained when using 2 mL of the sample; areas about 2% and 6% below were observed for the volumes of 5 and 8 mL of urine, respectively.

To establish extraction efficiency, 2,5-HD was diluted in carbon disulfide (solvent with negligible response to FID detector) in mass corresponding to a urine sample spiked with 5 mg/L of 2,5-HD. The corresponding area equivalent to the injection of the carbon disulfide solution was considered 100% for the test.

Results and Discussion

Chromatographic conditions

Figure 1 shows the chromatogram for HS-SPME-GC–FID of a urine sample spiked with 2,5-HD and the IS under the optimized conditions, showing the satisfactory resolution ($R = 2.99$) and efficiency (plate number = 14,464) for the analyte.

Selection of HS-SPME conditions in urine

Comparative evaluation of different fiber coatings. The choice of the fiber coating depends mainly on the nature of the target analytes (27). PDMS, CBW/DVB, CBX/PDMS, and PDMS/DVB fibers were evaluated. For this experiment, sampling time of 15 min, heating temperatures of 40 and 60°C, and desorption time of 5 min were chosen. A short sampling time is preferable for a first comparison among fibers when the coating is sampling by adsorption (PDMS/DVB, CBX/PDMS, CBW/DVB) instead of by absorption (PDMS) (28). CBX/PDMS fiber has greater affinity for 2,5-HD (100%), followed by the PDMS/DVB fiber (61.2%), PDMS fiber (1.42%), and the CBW/DVB fiber (0.75%) (Figure 2). On the other hand, better chromatographic efficiency was achieved by analyte desorption from PDMS/DVB fiber (plate number = 14,464) compared to that resulting from CBX/PDMS fiber (plate number = 1386); in addition, CBX/PDMS fiber requires a much higher desorption temperature (about 300°C) (29). Then, the PDMS/DVB was selected for the further experiments.

Sample volume. In HS-SPME, the sample volume is a variable directly related to the phase equilibrium (sample/HS/fiber). But the amount of extracted analyte will correspond directly to its concentration in the matrix, in spite of the sample volume (23). Three sample volumes were evaluated, 2, 5, and 8 mL of urine, taking into account the vial capacity (15 mL). Slight differences in absolute peak areas were observed, the best result being obtained when using 2 mL of the sample; areas about 2% and 6% below were observed for the volumes of 5 and 8 mL of urine, respectively.
**Effect of salt addition.** The extraction efficiency of SPME can also be influenced by the ionic strength of the sample matrix, which is known as the “salting-out effect” (27). The presence of salt increases the ionic strength of the matrix and often affects the solubility of analytes in biological samples. The effect of two types of salt (sodium chloride and sodium sulfate) was studied, as well as the best concentration of the salt. The best condition for extraction was achieved by the addition of Na₂SO₄, under stirring. Peak areas 5.2 times lower were observed when using NaCl. Sodium sulfate is a divalent ion which is usually more effective than univalent ions like sodium chloride for salting-out (29). A 1-g mass of Na₂SO₄ was used, enough to saturate the urine sample. An effective agitation is an important tool in analysis using HS-SPME to facilitate rapid extraction and transport of the analytes from the bulk of solution to the HS and vicinity of the fiber (23).

**Extraction and heating time and extraction temperature.** The extraction temperature and time are fundamental parameters for HS-SPME. The fiber equilibration process is exothermic, and any increase in sampling temperature will decrease both analyte recovery and equilibrium extraction time (30). HS/sample partition coefficient of the analytes increases with an increase in temperature, and at the same time, the fiber coating/HS partition coefficient decreases, hence the importance of the evaluation of these variables through factorial design. These designs are useful because it is possible to detect the most significant variables with few experiments. Preheating time was another parameter that could affect the equilibrium conditions.

According to the data from factorial design, extraction temperature and adsorption time had significant positive influences (i.e., higher temperature and adsorption time resulted in higher peak area for 2,5-HD; pre-heating time showed a very low negative effect). The second step was carried out to optimize the chosen significant variables using a central composite design (CCD). A 2² central composite design was performed for the two significant variables (extraction temperature and adsorption time) in order to refine the optimal conditions for urinary 2,5-HD HS-SPME extraction. The CCD has a star design added and the length of the arms of the star determined the number of levels and the shape of the experimental design. The CCD was completed by addition of a center point. The length of the arms of the star (α) played a major role in the appearance of the CCD. If α is different from 1, each variable will assume five levels (−α, −1, 0, +1, +α) (31).

The experimental design was constructed by the use of a full 2² factorial design with three central and four axial points (α = ±1.41), and the values for this second experiment are shown in Table I. This procedure offers an efficient route for determining the best resolution from a selected number of conditions (23).

Finally, the response surface plots were presented in order of visualization and rapid selection of optimal conditions. Response surface plots are presented in three-dimensional spaces and clearly show the influence of two factors (independent variables) that maximize the response of the dependent variable (2,5-HD peak areas) (Figure 3). According to the response surface obtained, the best conditions for HS-SPME analysis of 2,5-HD were extraction temperature, 50°C; and adsorption time, 20 min.

**Validation of the method**

After optimization, the methodology was validated according to internationally accepted criteria (32). The selectivity of the method was evaluated by analysis of blank matrices and blank matrices spiked with ethanol and acetone, common volatiles in urine samples. No interfering peaks from endogenous compounds were observed at the retention times of 2,5-HD and IS. Acetone and ethanol showed retention times far from those of the analytes, 2.1 and 1.3 min, respectively. The calibration curve was linear over the entire investigated range, 0.075–20.0 mg/L \(y = 0.044x + 0.015, R^2 = 0.995\). The LOD and LOQ of the method were 0.025 and 0.075 mg/L, respectively. These values are lower than published values for 2,5-HD determination by liquid–liquid extraction from urine, with LODs of 0.041 mg/L (GC–ECD) and 0.156 mg/L (GC–MS) (12), LOQs of 0.14 mg/L (GC–FID) (10), and LOD and LOQ of 0.05 and 0.10 mg/L, respectively (GC–FID) (14).

Table II summarizes the precision data obtained for the intra- and interassay precision studies. Although recovery studies are normally performed in bioanalytical method validation, the values are not normally reported in SPME studies (29). The extraction efficiency may give information

<table>
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<th>Variables</th>
<th>−1.41</th>
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<td>15</td>
<td>20</td>
<td>22</td>
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Figure 3. Response surface for HS-SPME urinary 2,5-HD extraction showing the area as a function of significant parameter extraction temperature (°C) and adsorption time (min), obtained using a 2² experimental design with CCD.
about recovery, and comparing the results of HS-SPME and direct injection in splitless mode is considered the technique of reference (33). The extraction efficiency of the proposed methodology was 7.3%. Extraction efficiency for volatiles in biological samples using HS-SPME is low compared to exhaustive methods of sample preparation such as liquid–liquid extraction. Values from 4.4 up to 14.6 for HS-SPME of benzene, toluene, ethylbenzene, and xylenes in urine have been reported (33). Therefore, the high enrichment factors of this miniaturized technique allow achievement of low LOQ values.

2,5-HD in urine of exposed individuals

The method was applied in order to analyze 2,5-HD in urine collected from eight workers applying glue to leather using a paintbrush. A calibration curve was constructed in a shorter range [i.e., from 0.075 to 2.5 mg/L (five points in quintuplicate each)], resulting in the linear regression equation \( y = 0.054x + 0.005 \) \((R^2 = 0.999)\), and it was used to quantify 2,5-HD in the samples. Levels between 0.08 and 0.99 mg/L 2,5-HD in urine were determined in these samples; only one of the results was above the biological exposure index proposed by ACGIH for free urinary 2,5-HD (BEI = 0.4 mg/L) (15).

Conclusions

The aim of this research was to develop and validate a method to determine 2,5-HD in urine samples based on HS-SPME extraction and GC–FID. The proposed method is simple, rapid, cost-effective, and solvent-free. The optimized HS-SPME technique in conjunction with GC–FID is reliable, precise, sensitive, and linear over a 0.075–20.0 mg/L range, and represents a useful tool for the biological monitoring of exposure to n-hexane through the determination of free urinary 2,5-HD.

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