Sensitive, rapid and simple method for multi-samples HPLC determination of urinary 2,5-hexanediione, suitable for automatic implementation

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ABSTRACT
In this paper we study the determination of urinary levels of 2,5-hexanediione (2,5-HD), an active metabolite of n-hexane by using high-performance liquid chromatography, DNPH derivatization and UV detection. We propose a novel method for 2,5-HD detection where the derivatization with DNPH is performed directly in the ethereal urinary extract. This allows to obtain only the mono-derivative of 2,5-HD which, injected in a C18 column, originates in the chromatogram a peak at 7 min. without interferences.

RIASSUNTO
Metodo sensibile, rapido e semplice per la determinazione multi-campione in HPLC del 2,5-esanodione, adatto per l’automazione
In questo lavoro viene studiata la determinazione della concentrazione minima di 2,5-esanodione (2,5-HD), un metabolite attivo del n-esano, mediante cromatografia liquida ad alte prestazioni derivatizzazione DNPH e rivelazione UV. Viene proposto un mono metodo per la rivelazione di 2,5-HD nel quale la derivatizzazione con DNPH viene eseguita direttamente nell’estratto etereo urinario. Ciò consente di ottenere solo in mono-derivato del 2,5-HD il quale iniettato in una colonna di C18 da luogo nel cromatogramma un picco a 7 minuti senza interferenza.

INTRODUCTION
Urinary 2,5-hexanediione (2,5-HD) is a biological indicator of occupational human exposure to n-hexane. It is a widely used solvent in paints, varnishes and glues, as well as in light petroleum and gasoline and utilised in shoe factories or leather industry.

Toxicological studies have shown that 2,5-HD is the active metabolite responsible of peripheral neuropathies leading to leg weakness progressing to paralysis (1-2).

The inhaled vapours of n-hexane are metabolized in the human organism to the 2,5-HD excreted in urine as free and as conjugated fraction. The urinary concentration of the 2,5 HD is closely related to n-hexane concentration in air; therefore the urinary excretion of 2,5HD is used to monitor occupational exposure to hexane (3-4).

Several methods have been developed methods for the detection of 2,5-HD in urine after hydrolysis (5).

In the indirect methods, after the acid hydrolysis step, a derivatization step is performed with dansylhydrazine (DNSH) or with 2,4-dinitrophenylhydrazine (DNPH) (12-13-14-15-16).

The DNSH derivatization procedure has several drawbacks: as solvent a toxic compound has to be used; the 2,5-HD derivative is a fluorescent compound and decays rapidly even at room temperature; and at least 30 min are necessary for sample (12).

On the contrary, the DNPH derivatization produces, through the condensation of one or two carbonylic groups, two stable compounds (mono or bis-derivative) that can be UV detected.

The mono-derivative of 2,5-HD shows higher solubility, a more significant UV response and a shorter retention time, shorter than the bis-derivative. The later possesses high molecular mass and strong interaction with the reversed phase column. The two hydrazones are synthetized from 2,5-HD aqueous solution with hydrazine reagent and identified in (16) by MS instrumentation.

In (16) the bis derivative elution is avoided by using a particular column and a buffer solution as a mobile phase.

However, the presence of several 2,4 - dinitrophenylhydrazones in the urine due to other compounds with carbonyl groups causes peaks that can interfere on the mono-derivative detection. For this reason, at least 30 minutes are necessary for eluting a single sample. Moreover, after...
each sample injection it is necessary to wash need and loop.

In this work we introduce DNPH derivatization process of 2,5-HD that allows to obtain exclusively the mono-derivative. The HPLC determination is then possible by using a C18 column and a mobile phase of acetonitrile and water.

The time per sample is only of about eight minutes and no cleaning is needed between consecutive samples. The proposed method is suitable for automatic implementation and therefore very useful for a routine determination.

MATERIALS AND METHODS

Hydrochloric acid, “HPLC grade” acetonitrile and diethyl ether were obtained by Merck (Darmstadt, Germany); 2,5-hexanedione (2,5-HD) and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Sigma (St. Luis, MO, U.S.A.). Deionized water was distilled in an all-glass still.

Chromatographic separation and peak detection of mono 2,4-dinitrophenylhydrazone of 2,5-HD were performed using a reversed-phase column C18, 5 µm packing, 150 x 4,6 mm I.D. from Varian (Harbour City, CA, U.S.A.) in a HPLC system consisting of a Series 9002 LC Pump and an LC 9050 ultraviolet spectrophotometer coupled to an integrator, all from Varian.

The conditions of HPLC separation were the following: mobile phase acetonitrile-water mixture (60:40, v/v); flow rate 1,0 ml/min; detection wavelength 330 nm; injection volume 20 µl. The urine specimens, kept in well closed bottles, were obtained from healthy, unexposed male subjects and workers exposed to n-hexane in the workshop.

Standard curve was prepared by adding known amounts of 2,5-HD (0 - 0.8 - 1.6 - 3.2 - 6.4 - 12.8 mg/l) to urine specimen of healthy unexposed male subject. All the urine specimens of exposed workers were collected at the end of a weekly shift between 4 and 6 p.m.; they were kept at -20°C for no more that 7-10 days, until analysis.

The DNPH solution was prepared by adding 250 mg of product to 100 ml of a 40/60 (v/v) hydrochloric acid and water mixture and by warming in a water bath at 60°C for 20 min.

At each 2,5 ml of urinary samples (obtained by standard curve and by workers exposed to n-hexane in the workshop) was added 250 µl of HCl. After vortexing, acid hydrolysis was achieved by incubation in an oven at 100°C for 1 h. After cooling at room temperature, 3,5 ml of diethyl ether was added to each specimen and the mixture vortexed in order to extract 2,5-HD.

The samples were centrifuged for 10 min at 300 rpm and 2.0 ml of each organic extract (the upper phase) was transferred to another test tube with a screw top.

The ethereal phase, after adding 50 µl of DNPH solution and 150 µl of distilled water, was dried at 37°C in a nitrogen stream. The remaining phase, after closing each tube with screw top, was warmed in a water bath at 60°C for 30 min.

20 µl of this solution was injected in HPLC system and peaks read at 330 nm.

The absence of interfering peaks allowed the identification of 2,5-HD by retention time (spiked peaks) and the quantification by an automatic peak height measure.

RESULTS

In this section, we show the results obtained by the present 2,5-HD determination method and compare them with those obtained by the derivation procedure in (16). The urinary specimen of a worker exposed to n-hexane was used to obtain two samples. One sample was derivatized with DNPH according to Gori et all. (16). The other one was derivatized according to the procedure described in the section on materials and methods.

The HPLC separation of both samples was performed by a C18 column at the conditions of mobile phase, flow rate, wavelength, injecting volume described in the present paper.

The figg. 1 and 2 show the chromatograms obtained...
by injecting the two samples prepared according to Gori et al. derivatization method (fig. 1) and to our derivatization method (fig. 2).

In fig. 1, mono and bis derivatives of 2,5-HD and an interfering compound are present. In this chromatogram the peak that spikes at about seven minutes corresponds to the 2,5-HD monohydrazone and the big peak after about twenty minutes corresponds to the bis-derivative.

This is not the case in fig. 2 where only the 2,5-HD mono-derivative peak appears. In fact under the reported derivatization conditions, it is possible to obtain exclusively the 2,5-HD mono-derivative as product of the reaction.

The Fig. 3 shows the calibration graph obtained by adding known amounts of 2,5-HD (0-0.8-1.6-3.2-6.4-12.8 mg/l) to urine specimen of a healthy unexposed man.

The calibration graph is linear up to 12.8 mg/l and can be represented by the regression equation $y = 11x$ were $y$ = peak height (mV) and $x$ = 2,5-HD concentration (mg/l).

The chromatogram in fig 4 shows the graph obtained by urine specimen of a worker exposed to n-hexane.

The limit of detection at a signal noise ratio of 2 is 0.1 mg/l. The within-run and between-run RSD’s were 4.8% and 9.6% respectively, using urine specimen (n = 20) containing 2,5-HD at the concentration of 3.2 mg/l. Urine samples of 20 not-exposed subjects showed a concentration lower than 0.1 mg/l. In the biological monitoring of workers exposed to n-hexane at the end of the shift and at the end of the working week, the 2,5-HD concentrations were lower than 0.4 mg/l, the biological exposure limit adopted in the year 2004 by American Conference of Governmental Industrial Hygienists (ACGIH, 17); only 9 urine samples from 62 workers range between 0.1 and 0.3 mg/l.

**DISCUSSION**

The proposed method represents an improvement with respect to Gori and all. method (16) for what concerns the speed of HPLC determination (a sample every 8 minutes). Also, no need and loop wash is necessary.

The more complex sample preparation phase is compensated by the possibility of preparing in parallel several samples (about 50 samples/2 hrs). The proposed method possesses the sensitivity needed to discriminate between exposed and unexposed people, it is linear up to quite high values. It shows an excellent reproducibility and moreover it uses very inexpensive reagents. The HPLC determination can be performed without the need of special equipment.

The proposed HPLC procedure is suitable for automatic implementation and therefor very useful when dealing with a large number of samples in biological monitoring of the workers exposed to n-hexane.
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