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Determination of 2,5-hexandione by high-performance liquid chromatography after derivatization with dansylhydrazine

L. Maestri, S. Ghittori, M. Imbriani*, E. Capodaglio

Centro Ricerche di Fisiopatologia e Sicurezza del Lavoro, University of Pavia, Clinica del Lavoro Foundation, IRCCS, Medical Center of Pavia, Via S. Boezio 24, 27100 Pavia, Italy

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Abstract

A sensitive method for the determination of free and total urinary 2,5-hexandione (2,5-HD) using high-performance liquid chromatography with fluorescence detection was developed. After purification of urine with a disposable C_{18} cartridge, 2,5-HD was derivatized with dansylhydrazine; 1,3-diacetyl benzene (1,3-DAB) was added to the samples, as internal standard, prior to extraction. The resulting fluorescent adducts were separated on a reversed-phase column with a gradient mobile phase of 25 m*M* phosphate buffer (pH 6.4) and acetonitrile. The retention times of the 2,5-HD and 1,3-DAB derivatives were 9.4 and 13.7 min, respectively. The derivatives were detected by a fluorescence detector (excitation 340 nm, emission 525 nm). The mean recoveries of 2,5-HD and 1,3-DAB were 92.0 and 94.0%, respectively; the detection limit of 2,5-HD (signal-to-noise ratio of 3) was 5 μg/l in urine without hydrolysis and *ca.* 12 μg/l in hydrolyzed samples. The method was applied to 39 urine samples from workers exposed to *n*-hexane; the mean values were 2.597 mg/l (S.D. = ±0.758) for total 2,5-HD and 0.179 mg/l (S.D. = ±0.086) for free 2,5-HD. Urine samples of 22 non-exposed subjects showed a mean concentration of 0.437 mg/l (S.D. = ±0.109) and 0.022 mg/l (S.D. = ±0.011) for total and free 2,5-HD, respectively.

1. Introduction

2,5-Hexandione (2,5-HD) is a common metabolite of *n*-hexane and methyl *n*-butyl ketone [1,2]; it has been shown to be directly responsible for neurotoxic effects in man and animals [3–8]. The urinary excretion of 2,5-HD is used to monitor occupational exposure to *n*-hexane, since the concentration of 2,5-HD in urine is related to the concentration of *n*-hexane in air. The quantitative relationship between the intensity of exposure to *n*-hexane and the urinary excretion of 2,5-HD is subject to variation due to possible metabolic interactions of *n*-hexane with solvents such as toluene and methyl ethyl ketone (MEK) under conditions of co-exposure.

At present, current methods for the determination of urinary 2,5-HD involve acid hydrolysis of the sample, followed by purification with disposable microcolumns; the sample is then extracted with dichloromethane and analysed by GC [9,10]. More recent studies reported that urinary 2,5-HD consists of a free fraction (that may be found in urine even without acid or enzymatic hydrolysis) and of a fraction arising from another metabolite of *n*-hexane, 4,5-

* Corresponding author.

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dihydroxy-2-hexanone, which is converted to 2,5-dimethylfuran at pH 2 and then to 2,5-HD by acid hydrolysis at pH 1 or less [11,12] (Fig. 1). Free 2,5-HD in urine constitutes approximately 10% of the total 2,5-HD [11,12].

The ACGIH (American Conference of Governmental Industrial Hygienists) [13] recommended a limit of 5 mg/g creatinine of 2,5-HD in urine for workers exposed to *n*-hexane (180 mg/m^3), provided that the urine was hydrolyzed at pH 2.0 for 30 min at 100°C. On the other hand, the German Research Society gave a threshold limit of 9 mg/l of 2,5-HD measured after hydrolysis at pH 1.0 or less [14].

According to some authors, there is no free 2,5-HD in the urine of unexposed subjects [15,16]; In contrast, analyses by GC–MS have revealed small amounts of this compound [12,17], probably arising from endogenous production of *n*-hexane, for example by lipid peroxidation. Urinary 2,5-HD is usually measured by gas chromatography [1,2]; a normal-phase HPLC technique with UV detection [18] and a procedure for the determination of 2,5-HD by reversed-phase HPLC with UV detection after its conversion to 2,5-dimethylpyrrole [19] have been proposed. The possibility to determine urinary 2,5-HD directly by reversed-phase HPLC-UV detection [20] is impracticable, due to the low molar absorption coefficient of 2,5-HD and to the relatively low amounts of this metabolite usually present in urine [21], particularly as a free fraction. To overcome these restrictions, we developed a method for the analysis of total and free 2,5-HD in urine by HPLC with fluorescence detection. The method involves the purification of samples by solid-phase extraction, followed by the reaction of 2,5-HD with dansylhydrazine (DNSH), a fluorescent reagent specific for carbonyl groups [22–24]. The method is suitable for routine biological monitoring of workers exposed to *n*-hexane; the high fluorescence of the 2,5-HD derivative makes it possible to measure total and free 2,5-HD even in unexposed subjects.

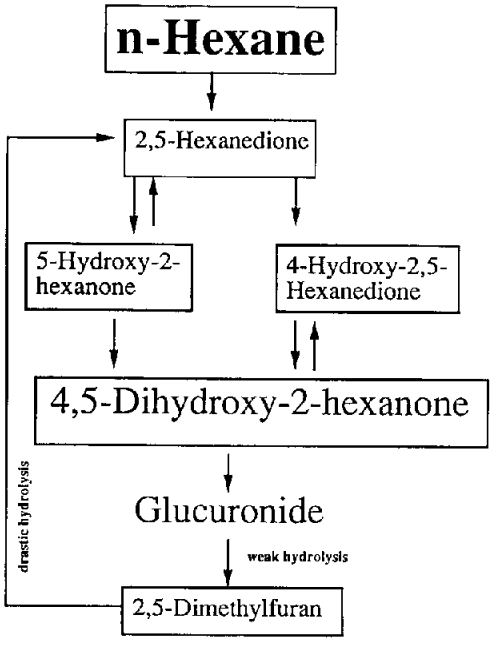

Fig. 1. Diagram showing the proposed production pathway of 2,5-HD from *n*-hexane and its metabolites.

2. Experimental

2.1. Chemicals

2,5-HD and 1,3-diacetylbenzene (1,3-DAB) were from Aldrich (Steinheim, Germany); DNSH was from Sigma (Munich, Germany). Acetonitrile and water were of HPLC grade (BDH, Poole, UK), while all other reagents were of analytical grade. Solid-phase extraction (SPE) ODS-silica cartridges (Bond-Elut C_{18}, size 3 ml) were from Analytichem International (Harbor City, CA, USA). Membranes used for mobile phase (0.22-μm) and sample (0.45-μm) filtering were from Millipore (Bedford, MA, USA). Stock solutions of 2,5-HD and 1,3-DAB in methanol could be stored at −20°C for at least one week without noticeable degradation, while the derivatizing reagent solution was prepared daily and filtered.

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA, USA) 600 E pump, a Jasco (Tokyo, Japan) 820-FP spectrofluorometer, a Waters WISP 715 autosampler and a NEC APC-IV

(Boxborough, MA, USA) computer for the acquisition and processing of the data. The analytical column was a Supelcosil (Supelco, Belafonte, USA) C_{18} 150 × 4.6 mm I.D. column, particle size 3 μm.

2.3. Pretreatment of samples

Urine samples (6.5 ml) were centrifuged for 10 min at 1000 *g*, and then 50 μl of the internal standard solution (1,3-DAB in methanol, 1.3 g/l) were added. Each sample was divided into two parts: 4 ml were directly applied to the SPE cartridge, while 2 ml were hydrolyzed (see below).

2.4. Hydrolysis

A 200-μl volume of 12 *M* HCl was added to 2 ml of urine, and the samples were incubated at 100°C for 45 min. After cooling at room temperature, samples were centrifuged (10 min at 1000 *g*) and extracted in the same way as the non-hydrolyzed urine.

2.5. Extraction

SPE cartridges were washed with 3 ml of methanol and 5 ml of 1.0 *M* HCl. A 4-ml volume of the non-hydrolyzed samples (or 2 ml of the hydrolyzed ones) was applied. The cartridges were washed with 5 ml of water and eluted with 2.5 ml of a mixture consisting of acetonitrile–water–phosphoric acid (50:50:2, v/v).

2.6. Derivatization

To 10 μl of purified sample 15 μl of the derivatizing solution (consisting of 30 mg of DNSH dissolved in 100 μl of dimethylformamide and 250 μl of acetonitrile) were added. After exactly 16 min at room temperature, 10 μl of the mixture were injected onto the HPLC apparatus. Each sample was automatically derivatized by the autosampler during the chromatographic elution of the previous sample.

Table 1
HPLC conditions for measuring 2,5-hexandione[a]

Time (min)	Curve	Mobile phase	
		% A	% B
12	Isocratic	40	60
3	Linear gradient up to	25	75
5	Isocratic	25	75
3	Linear gradient up to	40	60
7	Isocratic	40	60

[a] Column: C_{18}, 150 × 4.6 mm I.D., particle size 3 μm. Detector: fluorescence spectrophotometer; excitation 340 nm, emission 525 nm. Sample volume: 10 μl. Mobile phase: A = 25 m*M* phosphate buffer (pH 6.4), B = acetonitrile. Elution run: flow-rate = 1 ml/min.

2.7. Chromatographic analysis

Separation of the 2,5-HD and 1,3-DAB derivatives was performed by gradient elution with: solution A = phosphate buffer 25 m*M* (pH 6.4), solution B = acetonitrile, mixed at a constant flow-rate of 1 ml/min, according to the scheme shown in Table 1. Solvents were constantly degassed with helium during analysis. The retention times of the 2,5-HD and internal standard derivatives were 9.5 and 13.7 min, respectively. The spectrofluorometer was set at the wavelengths of 340 nm for excitation and 525 nm for emission.

3. Results and discussion

Dansylhydrazine is a fluorescent derivatizing reagent reacting with carbonyl groups [22–24]. The acid-catalyzed reaction between DNSH and a carbonyl group (Fig. 2) is usually performed for 10–30 min at 50–70°C [23,24]. In the case of 2,5-HD, however, we could not reveal the production of any derivative at these temperatures, probably due to the fast decay of the derivative. In the present work we studied the stability of the compound which originates from the reaction between DNSH and 2,5-HD at room temperature. Fig. 3 shows the decay of the fluorescent

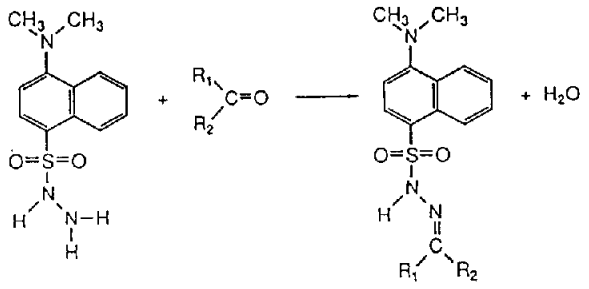

Fig. 2. Scheme of the reaction between DNSH and a carbonyl group.

derivative at room temperature under different conditions. The decay rate of the derivative depends also on temperature, in fact it is stable for at least 48 h when quickly frozen immediately after the reaction between 2,5-HD and DNSH. Because of the decay of the fluorescent derivative at room temperature, careful control of the reaction time is necessary, in a similar manner as for other derivatizing reagents used for chromatography (*e.g.* *o*-phtaldialdehyde, a well-known reagent specific for primary amines and amino-acids). Generally, when a derivatizing reagent has to be used for a compound contained in a complex matrix like urine, it is preferred to purify the samples in order to eliminate most of the substances containing derivatizable groups which could cause depletion of the reagent. In our experiments, when the above described extraction procedure was used, close linearity was found between the amounts of 2,5-HD added to urine and the fluorescence of the derivative up to 1 g/l of 2,5-HD. Moreover, the chromatographic peak of 2,5-HD showed no changes after the addition of 3 g/l of acetone (which also reacts with DNSH) to the purified samples. This demonstrated that DNSH was in large excess with respect to the total amount of carbonyl groups contained in the purified samples. DNSH is only slightly soluble in water [22],

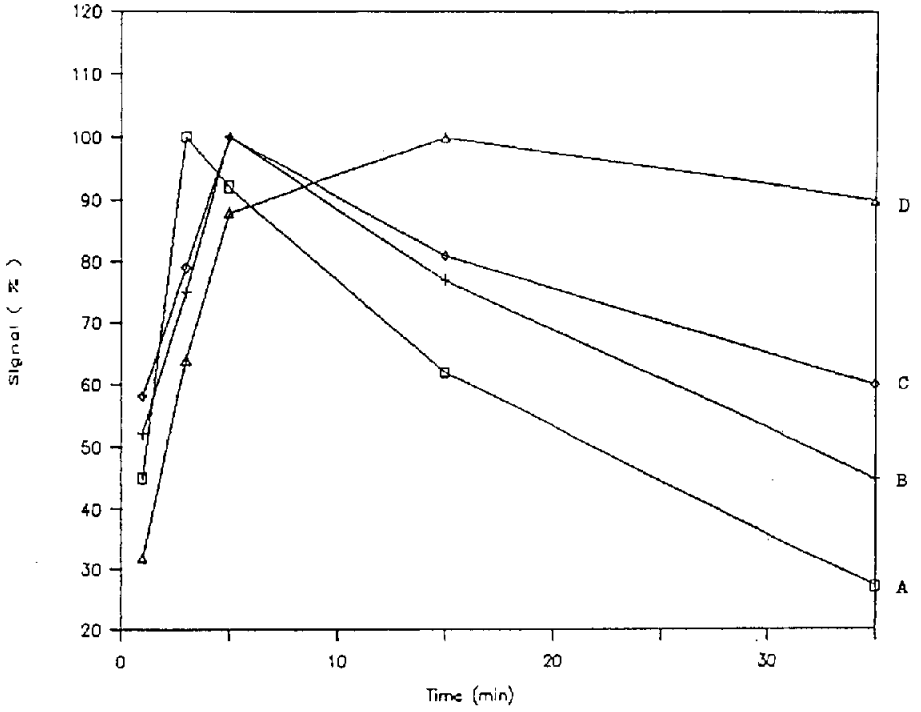

Fig. 3. Diagram showing the decay of the fluorescent derivative of 2,5-HD at different phosphoric acid and acetonitrile concentrations. H_3PO_4 and CH_3CN concentration (%): (A) 2.5/10, (B) 1/10, (C) 1/30, (D) 1/50. *x*-Axis = reaction time in min. *y*-Axis = % of the maximum signal. All curves were obtained by derivatization of standard 2,5-HD with DNSH at room temperature.

and thus the analyte must be dissolved in a matrix containing some organic solvent; an excess of organic solvent, however, may cause broadening of the chromatographic peaks [25]: the mixture used to elute the samples from the SPE cartridge (acetonitrile–water–phosphoric acid, 50:50:2, v/v) gave a good compromise between these two opposite requirements.

Authentic 2,5-HD (from 0.5 to 10 mg/l) was added to the same blank urine from a non-exposed subject; samples were then hydrolyzed, extracted and analyzed to obtain the calibration curve. The equation of the regression line was $y = 1.77 \cdot 10^6 x + 1.27 \cdot 10^5$; the correlation coefficient was $r = 0.9996$. The intercept on the y-axis was significantly different from zero and corresponded to a blank of approximately 0.200 mg/l of total 2,5-HD. To check the recovery, we added 2,5-HD (from 0.5 to 10 mg/l) and internal standard (10 mg/l) to seven randomly collected urines from exposed and non-exposed subjects, whose 2,5-HD concentration had been previously measured; samples were then processed as described. The recoveries were 92.0% for 2,5-HD and 94.0% for 1,3-DAB. The reproducibility of the results are summarized in Table 2.

Fig. 4 shows some examples of chromatograms. The peaks of 2,5-HD and 1,3-DAB appeared to have no interferences; the detection limit of 2,5-HD (at a signal-to-noise ratio of 3:1) was *ca.* 5 μg/l in the urine without hydrolysis. In the hydrolyzed samples the detection limit was higher (*ca.* 12 μg/l), due to the smaller amounts of urine applied to the SPE cartridges. The detection limit may be improved when the commercially available DNSH is purified by preparative chromatography on a silica column [25]: such a treatment reduces the baseline noise and allows higher volumes of sample to be injected (50 μl of derivatized sample).

In some commercial 2,5-HD standards Sturaro *et al.* [21,26] have found the presence of 3-methylcyclopenten-2-enone (3-MCP), a compound with high molar absorptivity that shows an HPLC behaviour similar to that of 2,5-HD; this compound may heavily interfere with the measurement of 2,5-HD by HPLC with UV detection [20,21]. In our chemical standard we also detected the presence of this impurity. In order to verify whether 3-MCP could interfere with the analysis of 2,5-HD by the present method, we purified these compounds from crude standard by means of semipreparative HPLC [21]. The two fractions were separately derivatized and injected (at comparable amounts) onto the analytical chromatographic system. Only the pure 2,5-HD fraction produced a peak with a retention time of 9.5 min. This confirmed the identity of the peak as 2,5-HD.

Every biological monitoring procedure of 2,5-HD in urine has to take into account that any acid treatment of urine leads to the conversion of 4,5-dihydroxy-2-hexanone to either 2,5-dimethylfuran (weak acid conditions) or 2,5-HD (strong acid conditions) [27]. In the latter case the concentration of 2,5-HD determined in urine

Table 2
Reproducibility of the determination of 2,5-HD in urine

Sample	Free 2,5-HD			Total 2,5-HD		
	Concentration[a] (mg/l)	C.V.[b] (%)		Concentration[a] (mg/l)	C.V.[b] (%)	
		Intra-assay	Inter-assay		Intra-assay	Inter-assay
1	0.046	9.1	10.3	0.320	4.1	4.8
2	0.195	6.3	8.0	1.283	3.7	4.8
3	0.403	4.5	6.8	3.950	3.4	4.5

[a] Mean of five determinations for each sample.
[b] Calculated on the basis of five determinations for each sample.

Fig. 4. Example of chromatograms obtained from the following samples: (A) blank (mixture CH_3CN–H_2O–H_3PO_4, 50:50:2, v/v), (B) standard mixture containing authentic 2,5-HD and 1,3-DAB, (C) urine sample (obtained from a subject exposed to *n*-hexane) without hydrolysis; the concentration of 2,5-HD was 0.270 mg/l, (D) the same as (C) after hydrolysis; the concentration of 2,5-HD was 3.085 mg/l. Further details on the analytical method are given in the text.

consists of more 2,5-HD arising artificially from 4,5-dihydroxy-2-hexanone than of original 2,5-HD [28]. In recent literature the total 2,5-HD detected in urine of subjects not occupationally exposed to MEK and *n*-hexane, is reported to have a mean value of 0.56 mg/l, a median of 0.6 mg/l and a range of 0.17–0.98 mg/l [17]. The levels of free 2,5-HD in urine of non-exposed subjects are below 50 μg/l. The detection limit of the present method makes it possible to determine the free and total 2,5-HD in urine samples from non-exposed subjects.

The present method was applied to 39 samples of urine obtained from *n*-hexane exposed workers; values ranged from 1.310 to 4.790 mg/l (mean = 2.597, S.D. = $\pm$0.758) for 2,5-HD after hydrolysis and from 0.06 to 0.405 mg/l (mean = 0.179, S.D. = $\pm$0.086) for free 2,5-HD. In contrast to previous studies [15,16] and in accordance with the studies reported by Perbellini *et al.* [17] and Fedtke and Bolt [12,28], the analysis of urine samples from subjects not exposed to *n*-hexane revealed the presence of 2,5-HD. Urine samples of 22 subjects showed a concentration between 0.185 and 0.732 mg/l (mean = 0.437, S.D. = $\pm$0.109) for total 2,5-HD, and between 0.010 and 0.048 mg/l (mean = 0.022, S.D. = $\pm$0.011) for samples without hydrolysis.

4. Conclusions

The present paper describes a method for the analysis of urinary 2,5-HD by HPLC with fluorescence detection, after purification of the samples with disposable SPE cartridges. The method is suitable for the measurement of both total and free 2,5-HD (with and without acid hydrolysis). Pretreatment of urine may be applied to a quite large number of samples, and the use of a suitable autosampler allows the samples to be automatically derivatized and injected. The procedure is sensitive, specific and fast enough to allow for routine analysis of this metabolite in urine of workers professionally exposed to *n*-hexane as well as of unexposed subjects.

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