

An improved method of analysing 2,5-hexanedione in urine

L Perbellini, D Marhuenda Amoros, A Cardona Llorens, C Giuliani, F Brugnone

Abstract

A short gas-chromatographic method for analysing urinary concentrations of 2,5-hexanedione is based on acid hydrolysis of urine at pH below 0.1 and "purification" of the urine samples by microcolumns containing an octadecyl-silane phase. A 5% acetonitrile solution allows a fairly selective elution of 2,5-hexanedione from the microcolumns. Recovery of 2,5-hexanedione from urine is as great as 79.9%. The variation coefficient of the measurements is 2.8%. The results obtained from different working conditions and using packed or wide bore or capillary gas-chromatographic columns are reported.

In a previous article we described a gas-chromatographic method for analysing the urinary metabolites of n-hexane, cyclohexane, and their isomers.¹ 2,5-Hexanedione (2,5-HD), 2-hexanol, γ -valerolactone, 2,5-dimethylfuran, 2-methyl-2-pentanol, 3-methyl-2-pentanol, and cyclohexanol could be analysed in the same urine sample. They can give information on the quality and on the level of exposures to aliphatic hydrocarbons with six atoms of carbon.

This method uses an enzymatic and an acid hydrolysis and is time consuming. Fedtke and Bolt obtained similar results using acid hydrolysis with urine at pH < 0.1^{2,3}; this procedure shortens the analysis time which may be further reduced if we analyse 2,5-HD only.⁴

Among aliphatic hydrocarbons, n-hexane, converted to 2,5-HD by physiological biotransformations, is certainly the most neurotoxic product. The urinary concentrations of 2,5-HD in urine of workers exposed to n-hexane are well correlated with the mean occupational exposure to the solvent during the

workshift.⁵⁻¹¹ Thus the analysis of urinary 2,5-HD is suggested for the biological monitoring of n-hexane exposures by ACGIH.¹²

This article summarises the best way to shorten the analysis time of urinary 2,5-HD and reports the different results that may be obtained with various gas-chromatographic columns.

Materials and methods

REAGENTS

The chemicals used were as follows: dichloromethane, cyclohexanone, acetonitrile (obtained from C Erba, Milan), 2,5-HD (from Eastman Kodak Co, Rochester, NY), and cartridges packed with 500 mg of octadecyl-silane phase (Bond Elut C18 from Analytical International Harbor City, CA).

URINE SAMPLES AND THEIR TREATMENT

Some samples of urine obtained from workers exposed to technical hexane in shoe factories were tested. The samples were frozen at -25°C until analysed. Five millilitres of urine put into glass tubes stoppered with Teflon screw caps were adjusted to pH lower than 0.1 by adding 1 ml of concentrated hydrochloric acid. Samples were heated at 100°C for 45 minutes in an oven and then allowed to cool to room temperature. The extraction of 2,5-HD was carried out by using cartridges prewashed by 3 ml of methanol and 5 ml of acid water (pH < 1). Then urine samples were applied: 2,5-HD was eluted with 3 ml of 5% acetonitrile/water solution. This solution was extracted using dichloromethane (2 ml) containing cyclohexanone (22 $\mu\text{g}/\text{ml}$) as internal standard by shaking for five minutes and then by centrifuging for one minute.

The recovered solvent was evaporated to 0.3 ml with a flow of nitrogen and used for the gas-chromatographic analysis.

The recovery rate of 2,5-HD was estimated by using four different concentrations of the metabolite: 0.9-2.9-6.8 and 9.7 $\mu\text{g}/\text{l}$. Three different samples of each concentration were used. After processing each sample according to the previous procedure, the recovery rate was calculated for comparison with the samples of 2,5-HD in dichloromethane containing

Institute of Occupation Medicine of the University of Verona, Policlinico Borgo Roma, 37134-Verona, Italy

L Perbellini, C Giuliani, F Brugnone
Department of Legal Medicine, University of Alicante, Alicante, Spain
D Marhuenda Amoros, A Cardona Llorens

Columns and operating conditions used to analyse urinary 2,5-HD

Column	ID (mm)	Film thickness (μm)	Length (m)	Oven operating conditions§		
				Initial temperature	Rate °C/min	Final temperature
SE 54*	0.32	0.5	50	80°C for 5 min	10°	200°C for 7 min
Carbowax 1500 0.2", on Carbo-pack 80/100 mesh	2	—	2	140°C for 5 min	2°	160°C for 15 min
CP Sil 19CB†	0.53	1	25	80°C for 6 min	5°	170°C for 4 min
HP5‡	0.1	0.17	10	90°C for 1 min	10°	140°C for 1 min

*Diphenyldimethylvinylpolysiloxano (from Supelco)

†Phenylmethylcyanopropylsilicone (from Chrompack)

‡Phenylmethylsilicone (from Hewlett Packard)

§Injector and detector temperature: 220°C.

the same amount of 2,5-HD and directly injected into a gas-chromatograph.

CALIBRATION CURVE

Standard solutions of 2,5-HD containing 0.9–2.9–6.8–9.7–30–50 and 100 mg/l were prepared in acid water; these solutions were put into prepared cartridges and treated as urine samples.

The reproducibility of the assay for the investigated metabolite was assessed by analysing 25 samples containing 2.9 mg/l of 2,5-HD. These analyses were done during five different days of two separate weeks.

INSTRUMENTATION

We used two different gas-chromatographs: a C Erba GC model. Fractovap 2350 with FID for packed or wide bore columns and a C Erba GC model 4160 with FID for capillary columns. Recorders were a Spectra Physics SP4270 or a Hewlett Packard model 3392A.

The GC/MS studies were carried out on a Hewlett Packard 5890 A GC connected with a HP 5970B mass selective detector with a SE 54 capillary column (table). These instruments were completed by a HP 5997AC chemstation. GC columns and the working conditions we used are reported in the table.

Results

During this investigation using our method we encountered aspects worth mentioning. The concentrated hydrochloric acid (1 ml) added to 5 ml of urine gives a pH always lower than 0.1. This aspect has been studied in more than 30 urine samples. The acid hydrolysis lasted 45 minutes: the recovery of 2,5-HD in urine samples containing more than 10 mg/l was lower if the urine heating was 30 minutes by comparison with heating lasting 45–90 minutes. The shaking time of extractions gave the same results when it ranged between five and 30 minutes.

The recovery of 2,5-HD from the acetonitrile/water solution using 2 ml of dichloromethane was equal to 79.9% (SD 5.9%). The amount retained by

cartridges was lower than 5% (with two or more subsequent extractions of the acetonitrile/water solution it is possible to obtain a higher recovery of 2,5-HD). The variation coefficient "intra-assay" was 2.8% and the variation coefficient "inter-assay" 2.9%. The calibration curve was linear in a range between 0.9 and 100 mg/l.

Figures 1–4 show the GC analyses of the same urine sample obtained from a worker exposed to

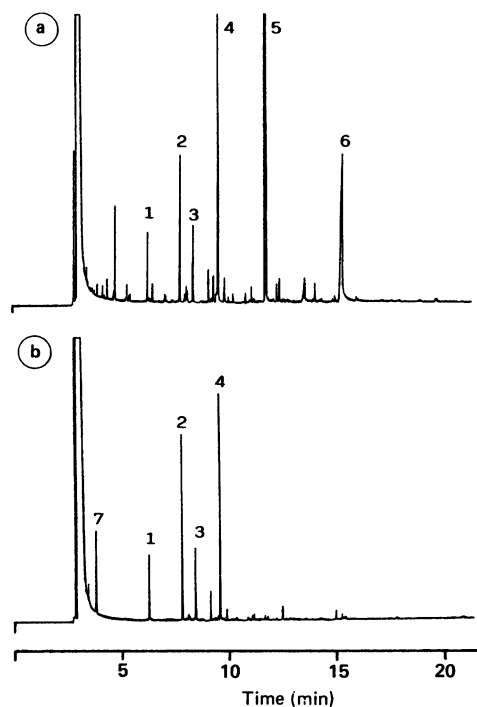


Figure 1 Gas-chromatograms obtained with the SE54 capillary column: solvent directly extracted from urine (a) or recovered from the acetonitrile/water solution (b). 1 = Disulfide dimethyl, 2 = cyclohexanone, 3 = 2,5-hexanedione, 4 = phenol, 5 = *p*-cresol, 6 = ethanone-1-(2-hydroxy-5-methylphenyl), 7 = silane, 1,4-phenylenebis (trimethyl).

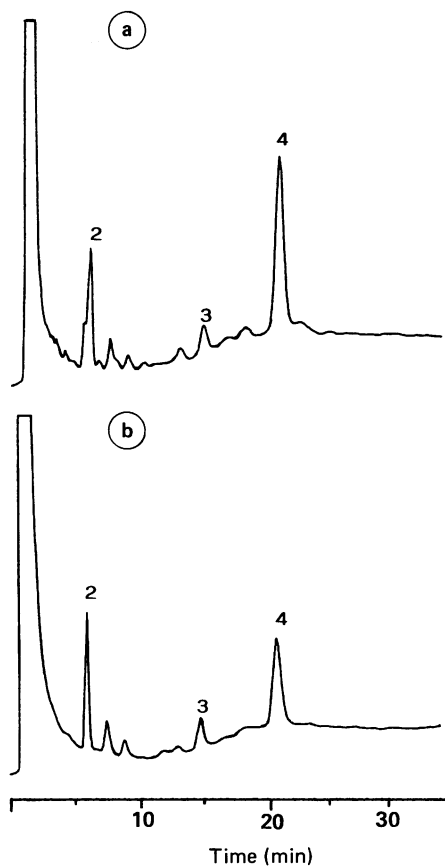


Figure 2 Gas-chromatograms obtained with the packed column: solvent directly extracted from urine (a) or recovered from the acetonitrile/water solution (b).

technical hexane. Figures 1–4a report the results using the solvent recovered from the direct extraction of the urine sample; figs 1–4b report the results using the solvent extracted after urine process with C18 silane phase. Figures 1a and 1b report the products identified by GC/MS using a SE 54 capillary column (table).

The main peaks present in the gas-chromatogram of the direct extraction (fig 1a) are cyclohexanone, 2,5-HD, phenol, and p-cresol. These products have been identified on the basis of GC retention time and their mass spectra were compared with those of authentic samples. Other products reported in fig 1a have been identified only by comparison with the spectra of NBS libraries stored in the HP chemstation. Most of these products and p-cresol are not present in the chromatogram of fig 1b, obtained by the solvent recovered after urine process with C18 silane phase. In this chromatogram the main peaks are represented by cyclohexanone, 2,5-HD, and phenol. The latter was present in a much lower

concentration than that in the direct extract of urine. The other two minor peaks present in the same figure are the silane, 1,4-phenylenebis(trimethyl) (probably derived by the C18 silane phase) and the disulphide dimethyl. (These products were identified by comparison of the data base spectra only.) Figures 1a and 1b suggest that the C18 silane phase retains most of the phenol, all the p-cresol, and numerous other products being present in small quantities. The analysis of the urine treated with C18 phase could be done in less time and it is not necessary "to purge" the gas chromatographic column by heating the oven to 200°C. The gas-chromatograms obtained with different columns may suggest different working methods.

Figures 2a and 2b underline that the 2,5-HD analysis with packed column can give reliable results. The analysis time reaches 25 minutes if we process the urine by the C18 silane phase. Otherwise we have to wait longer to purge the column from cresol and other products that remain in the column and threaten any further analysis.

With the wide bore column that we used (figs 3a and 3b), the 2,5-HD analysis may be completed in about 25 minutes if we use the direct extract. The

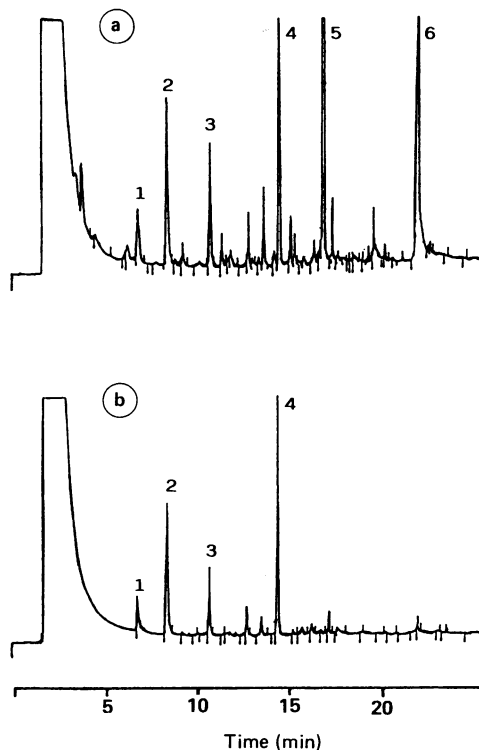


Figure 3 Gas-chromatograms obtained with the CP Sil 19CB column: solvent directly extracted from urine (a) or recovered from the acetonitrile/water solution (b).

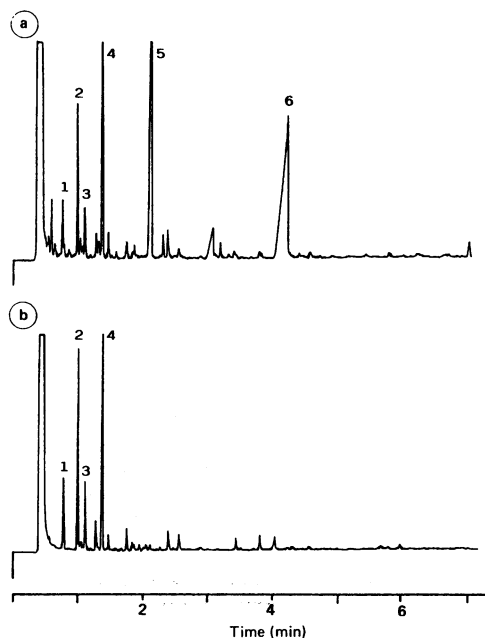


Figure 4 Gas-chromatograms obtained with the HP5 capillary column: solvent directly extracted from urine (a) or recovered from the acetonitrile/water solution (b).

extract of the eluted solution is clean enough and the analysis time is about 15 minutes. With a modified temperature programme the same analysis could be completed in 10 minutes and, in addition, it is not necessary to use a wide range of oven temperatures.

Figures 4a and 4b present the results obtained using a capillary column with an internal diameter of 0.1 mm. Its high separation capacity allows completion of our analyses in about four minutes if we use the direct extract of urine and in less than two minutes if we use the extract of the eluted solution.

Discussion

The methods for analysing urinary 2,5-HD previously described give similar results but they are quite time consuming.¹⁻³ Our analyses with different ways of extracting the 2,5-HD from the urine and with different gas-chromatographic columns allow completion of this analysis in different conditions, with different instruments, and in a shorter time.

If it is possible to work with the capillary column that gives results such as those reported in fig 4 the 2,5-HD analysis is completed in a few minutes. In this case the advantages of "clean" extracts are not important; perhaps it is cheaper to use the direct extracts because cartridges are not so necessary and you need not elute the urine with the C18 silane phase. By using other gas-chromatographic columns

we think that the use of C18 cartridges is useful and sometimes necessary (if you use packed columns).

2,5-HD was proved useful in the biological monitoring of n-hexane exposures by several authors.⁵⁻¹¹ Furthermore, ACGIH has introduced it in the biological exposure index.¹²

Fedtke and Bolt have reported that 4,5-hydroxy-2-hexanone is one of the main metabolites of n-hexane but it would be converted to 2,5-HD by urine acid hydrolysis.¹³ Perhaps the ratio between "free 2,5-HD" and "acid delivered 2,5-HD" will give further information on the pathogenesis of n-hexane neuropathies. The finding of Fedtke and Bolt, however, does not alter in any significant way the fact that 2,5-HD concentrations evaluated after urine acid hydrolysis still remains a useful and reliable method for obtaining information on the industrial exposure degree to n-hexane. Routine control of these exposures may be carried out reasonably rapidly using this method.

- 1 Perbellini L, Brugnone F, Silvestri R, Gaffuri E. Measurement of the urinary metabolites of n-hexane, cyclohexane and their isomers by gas chromatography. *Int Arch Occup Environ Health* 1981;48:99-106.
- 2 Fedtke N, Bolt HM. Methodological investigations on the determination of n-hexane metabolites in urine. *Int Arch Occup Environ Health* 1986;57:149-58.
- 3 Fedtke N, Bolt HM. Detection of 2,5-hexanedione in the urine of persons not exposed to n-hexane. *Int Arch Occup Environ Health* 1986;57:143-8.
- 4 Perbellini L, Tagliaro F, Maschio S, Zedde A, Brugnone F. Dosaggio gas-cromatografico del 2,5-esandione nelle urine (gas chromatographic measurement of urinary 2,5-hexanedione). *Med Lav* 1988;77:628-34.
- 5 Ahonen I, Schimberg RW. 2,5-Hexanedione excretion after occupational exposure to n-hexane. *Br J Ind Med* 1988;45:133-6.
- 6 Iwata M, Takeuchi Y, Hisanaga N, Ono Y. A study on biological monitoring of n-hexane exposure. *Int Arch Occup Environ Health* 1983;51:253-60.
- 7 Mutti A, Falzoi M, Lucertini S, et al. n-Hexane metabolism in occupationally exposed workers. *Br J Ind Med* 1984;41:533-8.
- 8 Arfaioi C, Bavazzano P, Biancalani P, et al. Utilizzazione del 2,5-esandione urinario nel monitoraggio biologico degli esposti a n-esano. 48 Congr Naz Soc Ital Med Lav Igiene Ind Pavia 1985. (Urinary 2,5-hexanedione as indicator of n-hexane exposure. Proceedings of the 48th National Congress of Italian Society of Occupational Medicine and Industrial Hygiene.) Bologna: Monduzzi Ed, 1985.
- 9 Bartolucci GB, Perbellini L, Boscaro G, et al. Monitoraggio biologico dell'esposizione a n-esano nel settore calzaturiero. 50° Congr Naz Soc Ital Med Lav Igiene Ind Rome, 1987. (Biological monitoring of shoe factory workers exposed to n-hexane. 50th National Congress of the Italian Society of Occupational Medicine and Industrial Hygiene.) Bologna: Monduzzi Ed, 1985.
- 10 Perbellini L, Bartolucci GB, Brugnone F, De Rosa E, Valentini F. Il 2,5-esandione nel controllo biologico dell'esposizione professionale a n-esano. (Biological monitoring of workers exposed to n-hexane using the 2,5-hexanedione) *Med Lav* 1985;76:35-43.
- 11 Perbellini L, Brugnone F, Faggionato G. Urinary excretion of the metabolites of n-hexane and its isomers in occupational exposure. *Br J Ind Med* 1981;38:20-6.
- 12 American Conference of Governmental Industrial Hygienists. *Threshold limit values for chemical substances and physical agents in the work environment and biological exposure indices with intended changes for 1985-6*. Cincinnati: ACGIH, 1987.
- 13 Fedtke N, Bolt HM. The relevance of 4,5-dihydroxy-2-hexanone in the excretion kinetics of n-hexane metabolites in rat and man. *Arch Toxicol* 1987;61:131-7.