Journal of Chromatography, 592 (1992) 255–260
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2446

Rapid and sensitive determination of urinary 2,5-hexanedione by reversed-phase high-performance liquid chromatography

M. Perla Colombini

Dipartimento di Chimica e Chimica Industriale, Via Risorgimento 35, 56100 Pisa (Italy)

Patrizio Carrai

Dipartimento di Tossicologia, Ospedale Campo di Marte, U.S.L. 6, Lucca (Italy)

Roger Fuoco* and Carlo Abete

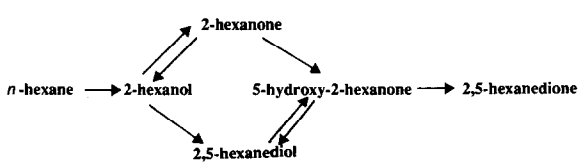
Istituto di Chimica Analitica Strumentale del CNR, Via Risorgimento 35, 56100 Pisa (Italy)

ABSTRACT

A rapid and sensitive method for the determination of 2,5-hexanedione (HD) (the principal metabolite of *n*-hexane) in urine samples by reversed-phase high-performance liquid chromatography (HPLC) is described. The sample preparation procedure was based on solid–liquid extraction after acid hydrolysis; it was optimized to enable accurate HD determination in less than 30 min. Analysis of spiked real samples showed a recovery of more than 85% at the 0.1-ppm level, with a relative standard deviation of 5% and a detection limit as low as 0.01 ppm. Intra-assay and inter-assay coefficients of variation at the 0.5-ppm level were 4 and 5%, respectively. The chromatographic peak assigned to HD was identified by collecting the HPLC eluate at the retention time of HD and analysing it using Fourier transform infrared spectrometry coupled with high-resolution gas chromatography. Urine samples of unexposed and exposed subjects were analysed following the proposed analytical procedure. HPLC and high-resolution gas chromatographic analyses were also compared on these samples. A correlation factor of 0.992 was obtained, which showed a good agreement between the two sets of data.

INTRODUCTION

n-Hexane is a widely used solvent contained in paints, varnished and glues, as well as in petroleum ether and gasoline. Occupational exposure to it causes polyneuritis and pheripheral neuropathies which manifest themselves as leg weakness leading to paralysis [1,2]. Absorption of *n*-hexane in exposed workers occurs almost entirely through the respiratory system [3,4] and follows this biotransformation cycle:



Its neurotoxic effect has been attributed to the metabolite 2,5-hexanedione (HD), which may be bonded to DNA, RNA and essential proteins [4,5]. Studies [6–8] on the urinary excretion of the *n*-hexane metabolite HD, determined in exposed factory workers, have shown a correlation between the metabolite and *n*-hexane concentrations in the work-

0021-9673/92/$05.00

room air. Owing to the neurotoxic effects of HD, which are closely related to the concentration of *n*-hexane in air, it is advantageous to have a rapid and sensitive method which can determine this compound in urine samples for biological monitoring of occupational exposures, even at very low levels.

High-resolution gas chromatography (HRGC) [8–11] has been proposed for the determination of urinary HD levels. Acid hydrolysis [9–12] has significantly lowered the time of sample preparation compared with the enzymatic method [8]; and solid–liquid extraction, with octadecylsilane microcolumns [9–11], has made quantitative analysis by HRGC much less time-consuming than extraction with solvents [9,12].

In the analytical procedure for reversed-phase high-performance liquid chromatography (HPLC) reported in the literature [12] the sample is subjected to liquid–liquid extraction, and the extract is evaporated to dryness and reconstituted with water before chromatographic analysis is performed. This is quite time-consuming and, moreover, the drying step may cause some irreproducible loss of HD at low concentration levels. Reliability at these levels is important, for example to evaluate low urinary HD levels in subjects exposed to small amounts of solvent who may suffer long-term effects as a result of chronic poisoning.

This paper describes a rapid and sensitive procedure for the determination of HD in urine samples by reversed-phase HPLC. The extraction of HD from the sample and the clean-up of the extract are based on solid–liquid extraction. Commercial microcolumns were used. Elution solvent sequences along with elution volumes and times were optimized for better results and shorter analysis times. The optimized procedure reduces sample preparation to less than 15 min, leading to a very low detection limit (0.01 ppm) with a typical recovery of about 85% and a relative standard deviation (R.S.D.) of 5% at the 0.1 ppm level. Results obtained on real samples collected from exposed and unexposed subjects, and the correlation between HRGC and HPLC measurements, are also discussed. Fourier transform infrared (FTIR) spectrometry, coupled with HRGC by a cooled moving-plate interface developed in our laboratory [13–15], was employed for confirming the identity of the chromatographic peak assigned to HD.

EXPERIMENTAL

Reagents

HPLC-grade organic solvents were supplied by either Carlo Erba (Italy) or Riedel-deHaen, (Germany). Sodium hydroxide and hydrochloric acid were Suprapur reagent grade (Merck, Germany); sodium carbonate, cyclohexanone and *trans*-2-butenal were analytical reagent grade (Carlo Erba). 2,5-Hexanedione was supplied by Aldrich Chimica (Italy). Standard solutions of 2 g/l were prepared weekly in dichloromethane, methanol or water and stored at 4°C. Anydrous sodium sulphate (Carlo Erba) was kept at 450°C for 6 h, stored in a dessicator and washed with dichloromethane before use. Double-distilled water (Carlo Erba) was treated in an ELGASTAT UHQ system to produce ultrahigh-quality pure water (18 MΩ/cm resistivity at 25°C) and was used throughout.

Octadecyl spe (C_{18}, J. T. Baker, USA) and Supelclean LC-Si spe (LC-Si, Supelco, USA) 3-ml cartridges were used for HD extraction and extract clean-up, respectively. Millex HV_{13} filter units (0.45 μm, Millipore, USA) were used for filtering urine samples.

Apparatus

An LC-410 series HPLC system (Perkin Elmer, USA) equipped with a Reodyne Model 7125 injector (20 μl injection loop), an LC-95 UV–VIS spectrophotometric detector and a C-R3A chromatopac integrator (Shimadzu) was used for LC analysis. Chromatographic separation was performed on an S5ODS1 C_{18} reversed-phase spherisorb column (Phase Separation, USA), 250 × 4.6 mm I.D., 5 μm particle size. Isocratic elution was carried out by 15% acetonitrile in water at a flow-rate of 1 ml/min (overall time for one run: 12 min). The spectrophotometric detector was set at a wavelength (λ) of 230 nm, where HD exhibits the maximum absorbance.

An HRGC-5160 Mega series chromatograph (Carlo Erba) equipped with an automatic cold on-column injection port, a flame ionization detector heated at 250°C and a Hewlett-Packard Model 3396A integrator was used for GC analysis. Chromatographic separation was performed on a fused-silica Series 007 capillary column (Quadrex, USA), methyl phenyl 5% silicone, 25 m × 0.32 mm I.D., 0.25 μm film thickness. The chromatographic con-

ditions were: initial temperature, 40°C; 1°C/min increase up to 50°C and 25°C/min increase up to 250°C; and hold for 4 min. Helium was used as the carrier gas.

A Model 740 FTIR spectrometer (Nicolet, USA) equipped with a narrow-range 1-mm square element mercury–cadmium–telluride (MCT) detector and a Model Spectra-Scope microscope unit (Spectra-Tech, USA), with a variable aperture from 20 μm to 15 mm, was coupled to the HRGC system by a moving-plate interface [13–15] and was used for collecting 8 cm^{-1} resolution IR spectra.

Procedure

The overall analytical procedure proposed was as follows: load the C_{18} column with 5 ml of urine (pH 2); wash with 3 ml of water, 3 ml of 0.1 *M* sodium carbonate and 6 ml of water; elute with 1.5 ml of dichloromethane; dry on sodium sulphate; load the LC-Si column with dichloromethane eluate; wash with 1 ml of dichloromethane and 1 ml of diethyl ether, 25% in dichloromethane; elute with 1 ml of methanol.

Urine pretreatment. Following a slightly modified version of a procedure proposed by Perbellini *et al.* [11], urine (20 ml) was acidified with concentrated hydrochloric acid (1 ml) and kept in a closed vessel at 100°C in an oven for 1 h. After cooling, the pH was adjusted to 2 by adding concentrated sodium hydroxide solution, and urine was filtered by using a Millex filter unit and immediately processed. A series of experiments carried out on spiked urine in which the pH was varied from 0 to 7 showed that HD losses were more than 80%, except for pH ranging between 1.5 and 3.5.

Cartridge preparation. Octadecyl spe cartridges were preconditioned immediately before use by washing them under vacuum with methanol (6 ml), water (3 ml) and hydrochloric acid at pH 2 (3 ml).

LC-Si spe cartridges were preconditioned by washing them only with dichloromethane (6 ml). A 2-g sample of anydrous sodium sulphate was put on the top of the LC-Si column to eliminate water traces which may have been present in the dichloromethane eluate.

HD extraction and extract clean-up. A 5-ml sample of pretreated urine was loaded on an octadecyl spe cartridge and washed with 3 ml of water, followed by 3 ml of 0.1 *M* sodium carbonate solution and 6 ml of water. This washing procedure was needed in order to eliminate most of the organic substances which may cause severe interferences in the chromatographic determination. HD was eluted with 1.5 ml of dichloromethane by pumping with a gas–tight glass syringe. Before eluting, the cartridge containing HD was dried under mild vacuum. The results showed low recoveries and high standard deviations, and very strict experimental conditions had to be respected. This was explained by considering the quite high volatility of HD and the stripping out process from the cartridge. Therefore, the solvent was dried on anydrous sodium sulphate.

HPLC analysis of dichloromethane extract, after evaporating to dryness under argon flow and reconstituting with methanol highlighted the fact that some interfering substances were still present. In order to remove them, a final clean-up was done by loading dichloromethane extract on an anydrous sodium sulphate LC-Si spe cartridge, washing with 1 ml of dichloromethane, followed by 1 ml of diethyl ether, 25% in dichloromethane, and eluting HD with 1 ml of methanol by pumping with a gas-tight glass syringe. A total of 0.5 ml of eluate was collected, 20 μl of which were injected into the HPLC system for HD determination. For low HD content, the extract was concentrated to 0.1 ml in a micro-Kuderna-Danish evaporator under argon flow at room temperature before being injected.

HRGC analysis procedure. For the sake of comparison, HRGC analysis was performed on the dried dichloromethane extract of some urine samples processed following the proposed procedure. Quantitation was done by using calibration plots obtained with spiked real samples.

Internal standard. trans-2-Butenal and cyclohexanone were used as internal standards (I.S.) for HPLC and HRGC, respectively. In both cases, these compounds elute before HD, having been sufficiently resolved from the HD and from other peaks present in real samples. Cyclohexanone and *trans*-2-butenal were added directly to dichloromethane or methanol extract, respectively.

RESULTS AND DISCUSSION

Quantitation and detection limits

In biological samples, metabolic products may interfere with the determination of the compound

under investigation. In order to take into account any possible interferences, quantitative HPLC determinations of HD were performed using calibration curves obtained by means of a calibration solution prepared from urine samples collected from unexposed subjects which were tested in advance and showed a non-detectable HD level. Spiked samples, with a concentration range of 0.1—50 ppm, were processed following the proposed procedure. Linear regression analysis for the 95% confidence limit of all data collected gave the formula $y = 0.70 + 37.05\,x$ ($r = 0.9998$), where y is the peak area in arbitrary units and x is the concentration in ppm. The following remarks can be made from these results: (i) the calibration curve is linear in the observed concentration range and shows a fairly high correlation coefficient (r); (ii) the intercept value expressed in ppm and the relative error on the slope are typically 0.02 and 1%, respectively.

To calculate the detection limit (c_L), the following definition, recommended by IUPAC [16], was used:

$$c_L = k\,s_B/S \qquad (1)$$

where k is a numerical constant for which IUPAC strongly suggests a value of 3, s_B is the standard deviation of the field blank and S is the sensitivity of the method. Since, in the present case, true field blanks were not available, s_B was considered to be equivalent to the standard deviation (σ_0) obtained by extrapolating at zero concentration the standard deviations on seven replicate analyses of five spiked samples at low concentration levels (0.1–0.5 ppm). From the calculated σ_0 values, a detection limit of 0.05 ppm using eqn. 1 was obtained. Moreover, since organic extracts can be reduced to a volume of 0.1 ml under argon flow at room temperature, the detection limit can be lowered to 0.01 ppm. Replicate analysis on the same sample containing 0.01 ppm showed a relative standard deviation of 31%.

To calculate the concentration of the sample, the measured peak area was first corrected for the I.S. response and then converted into ppm by means of the slope of the corresponding calibration plot. The urinary concentrations of HD were finally corrected to a specific gravity of 1024.

Recovery and precision

The reliability of the present method was tested by using spiked urine samples prepared as outlined earlier. Triplicate samples of urine were spiked with various amounts of HD in the range 0.1–50 ppm and analysed using the proposed method. The results for HPLC analysis are reported in Table I. These data show recoveries of more than 85% for concentrations as low as 0.1 ppm.

TABLE I

RECOVERY OF HD FROM SPIKED URINE SAMPLES

Concentration (ppm)	Recovery[a] (%)	
	Mean	R.S.D.
0.1	85	5
0.2	86	4
0.4	85	4
1.0	89	3
5.0	88	3
10.0	92	2
50.0	94	2

[a] Three replicate measurements.

In order to improve the validation of the analytical procedure proposed, three more calibration solutions containing 0.5, 1.0 and 1.5 ppm HD were prepared. These calibration solutions were analysed ten times per day, on three non-consecutive days, interspersing them with urine samples collected from exposed and unexposed subjects. Thus it was possible to obtain intra-assay and inter-assay statistical figures on ten and thirty pieces of data, respectively. The results are reported in Table II and show that at the 0.5-ppm level the coefficient of variation

TABLE II

INTRA-ASSAY AND INTER-ASSAY COEFFICIENT OF VARIATIONS FOR HD DETERMINATIONS

Concentration (ppm)	Coefficient of variation (%)	
	Intra-assay[a]	Inter-assay[b]
0.5	4	5
1.0	3	4
1.5	3	4

[a] Ten replicate measurements.
[b] Thirty replicate measurements.

is always more than 5%, even for inter-assay analysis.

One of the urine samples analysed, collected from a subject not known to have been exposed to *n*-hexane, showed a signal at the HD retention time whose concentration was estimated to be 0.15 ppm. To confirm its identity the following procedure was used: recovery of the HPLC fraction eluted at HD retention time, liquid–liquid extraction with dichloromethane, reduction to 0.02 ml in a microKuderna-Danish evaporator under argon flow at room temperature and analysis by HRGC–FTIR. The cooled moving-plate interface described elsewhere [13–15] was kept at −40°C during the trapping of HD on its surface. IR transmission spectra collected after a 1-μl injection of a standard solution containing 50 ng/μl HD and after a 1-μl injection of concentrated dichloromethane solution showed the same features, so the assignment to HD of the peak obtained at 9.15 min retention time is correct.

Real samples

Urine samples were collected from shoe factory employees working in different environmental conditions following the procedure proposed in ref. 6. All samples were divided into two groups: weakly exposed and exposed to vapours of *n*-hexane, depending on the exposure level. Control samples were collected from unexposed voluntary subjects. For each group, four samples were collected from four different subjects and analysed in triplicate. For the first two groups the maximum concentration of *n*-hexane in the workroom air was estimated to be about 20 and 200 mg/m^3, respectively. The only purpose of the sample analysis was to verify the ability of the proposed analytical procedure to determine also low levels of HD which occur after occupational exposure to *n*-hexane, and to compare HPLC and HRGC data.

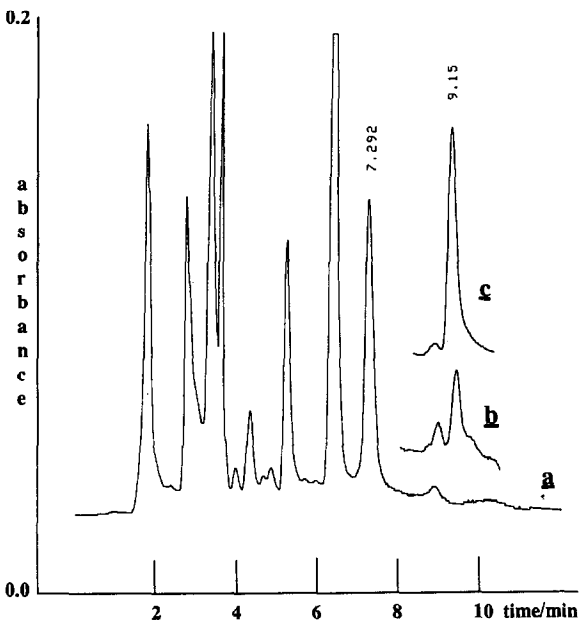


Fig. 1. HPLC chromatograms for samples of unexposed (a) and weakly exposed (b) workers. Curve c refers to sample b with a 0.3-ppm standard addition of HD. Signals at 7.29 and 9.15 min relate to I.S. and HD, respectively.

HPLC chromatograms of samples from an unexposed and an exposed subject are shown in Fig. 1. Curve a reveals undetectable HD concentrations in one sample of an unexposed subject. This chromatogram also shows that in the time intervals where the signal under consideration falls, the baseline is quite flat and unaffected by the presence of possible interferences. For one sample of a weakly exposed subject (curve b) the HD concentration was estimated to be 0.10 ppm. Curve c refers to another aliquot of the sample b after an addition of 0.3 ppm HD.

The results for all the real samples analysed by

TABLE III

HD CONCENTRATION IN REAL SAMPLES

Subject group	Concentration (ppm)	
	HRGC	HPLC
Exposed	3.80	3.50
	1.10	1.30
	1.80	2.00
	2.80	2.40
Weakly exposed	0.15	0.16
	0.45	0.40
	0.12	0.10
	0.22	0.28
Unexposed	n.d.	n.d.
	0.13	0.15
	0.08	0.05
	0.03	0.04

both HPLC and HRGC are summarized in Table III. Linear regression analysis between two sets of data was performed, and a correlation factor of 0.992 was obtained, which shows that there is good agreement between the HPLC and HRGC results. The data in Table III also demonstrate a fairly high variability of the HD urinary level within the first and second groups. This expected variability may be explained by considering the differences in exposure time and level of exposure to *n*-hexane between subjects from whom samples were collected. Finally, small amounts of HD were found in some urine samples of unexposed subjects (third group) for whom a non-detectable level should be expected. The occurrence of these low HD levels may be the result of an occasional exposure to *n*-hexane or of other unknown metabolic reactions which may induce urinary HD [12,17].

CONCLUSIONS

An overall analytical procedure is described for a sensitive and accurate determination of HD urinary level. Sample preparation was optimized to enable HPLC analysis in a short time. After acid hydrolysis, the overall analysis time was less than 30 min. The proposed procedure is reliable and can be used for a routine analysis with an intra-assay coefficient of variation of 4% and inter-assay coefficient of variation of 5% even at the 0.5-ppm level. HPLC and HRGC procedures were performed on several urine samples and compared, and gave a correlation factor of 0.992. The detection limit is about 0.01 ppm, so the presence of HD in urine samples can also be estimated for workers exposed to very low quantities of *n*-hexane.

ACKNOWLEDGEMENT

One of the authors (M.P.C) thank the M.P.I. for financial support.

REFERENCES

1 M. Iwata, Y. Takeuchi, N. Hisanaga and Y. Ono, *Int. Arch. Occup. Environ. Health*, 51 (1983) 253.
2 H. H. Schawmburg and P. S. Spencer, *Brain*, 99 (1976) 183.
3 D. Couri and M. Milks, *Am. Rev. Pharmacol. Toxicol.*, 32 (1982) 145.
4 R. Toftgard and J. Gustafsson, *Scand. J. Work Environ. Health.*, 6 (1980) 1.
5 A. P. De Caprio and E. A. O'Neil, *Toxicol. Appl. Pharmacol.*, 78 (1985) 235.
6 I. Ahonen and K. W. Schimberg, *Br. J. Ind. Med.*, 45 (1988) 133.
7 H. Mat and H. Kenneth Dillon, *Biological Monitoring of Exposure to Chemicals*, Wiley, London, 1987.
8 L. Perbellini, F. Brugnone and G. Faggionato, *Br. J. Ind. Med.*, 38 (1981) 20.
9 L. Perbellini, F. Tagliaro, S. Maschio, A. Zedde and F. Brugnone, *Med. Lav.*, 77 (1986) 628.
10 N. Fedtke and H. M. Bolt, *Int. Arch. Occup. Environ. Health*, 57 (1986) 149.
11 L. Perbellini, D. Marhuenda Amoros, A. Cardona Llorens, C. Giuliari and F. Brugnone, *Br. J. Ind. Med.*, 47 (1990) 421.
12 I. Marchiseppe, M. Valentino, M. Governa and V. Stocchi, *J. Chromatogr.*, 492 (1989) 288.
13 K. H. Shafer, P. R. Griffiths and R. Fuoco, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 124.
14 R. Fuoco, K. H. Shafer and P. R. Griffiths, *Anal. Chem.*, 58 (1986) 3249.
15 R. Fuoco, in S. Caroli, G. Rossi, E. Sabbioni and K. Zimmer (Editors), *Biomedical Research and Spectrochemistry*, C.E.C. No. EUR 11702 EN, Brussels, 1989, p. 203.
16 Analytical Methods Committee, *Analyst*, 112 (1987) 199.
17 H. Altenkirch, G. Stoltenburg and H. M. Wagner, *J. Neurol.*, 219 (1978) 159.