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# Note

## Determination of 2,5-hexanedione in urine and serum by gas chromatography after derivatization with O-(pentafluorobenzyl)hydroxylamine and solid-phase extraction

S. KEŽIĆ and A. C. MONSTER*

*Coronel Laboratory for Occupational and Environmental Health, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam (The Netherlands)*

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*n*-Hexane is a solvent widely used in industry, especially in glues, varnishes and paints. Occupational exposure has been reported to cause peripheral neuropathies in workers [1,2]. The toxicokinetics of *n*-hexane has been widely investigated in rats, but much less is known in humans. *n*-Hexane is metabolized by P-450-dependent monooxygenases and alcohol dehydrogenases to a series of metabolites, including 1-hexanol, 2-hexanol, 3-hexanol, 2-hexanone, 5-hydroxy-2-hexanone, 2,5-hexanedione (25HD) and 4,5-dihydroxy-2-hexanone (45DH2H) [3,4]. 2-Hexanone, a metabolite of *n*-hexane, is also a widely used solvent in industry. Toxicologically, the most interesting metabolite of *n*-hexane is 25HD, because it is the primary cause of *n*-hexane-induced peripheral neuropathy. Presumably it forms substituted pyrole adducts with axonal proteins [5,6].

The determination of 25HD has been proposed for biological monitoring of persons occupationally exposed to *n*-hexane; the established biological exposure index in the United States is 5 mg/l [7] and in F.R.G. 9 mg/l [8].

The metabolites of *n*-hexane are usually determined after acid hydrolysis of urine at 100°C, followed by solvent–solvent extraction [9] or solid-phase extraction [10]. However, Fedtke and Bolt [11,12] have recently pointed out that acid hydrolysis treatment leads to the conversion of 45DH2H into either 25HD or 2,5-dimethylfuran, depending on the pH of the urine. The concentration of 25HD with acid hydrolysis in rat urine after exposure to *n*-hexane was about ten times the amount found without acid hydrolysis. This implies that reported biological exposure indices [7,8] refer, in fact, to the sum of 25HD and 45DH2H. Since 25HD as such is supposed to be responsible for the toxic action of *n*-hexane, any kind of artificial increase in its concentration must be avoided. For that reason a more sensitive method for the determination of actually present 25HD in urine and blood than those available was needed.

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## EXPERIMENTAL

### *Principles*

In order to improve the extraction recovery and sensitivity of the determination, 25HD was derivatized with O-(pentafluorobenzyl)hydroxylamine (O-PFBHA) to the corresponding oxime. The derivatization was carried out in urine or serum samples. Since the reaction with O-PFBHA proceeds readily also in weakly acidic media (pH 4–6) [13], pH adjustment of urine and serum samples was unnecessary. The derivate of 25HD was extracted from urine using solid-phase extraction. Gas chromatography (GC) was used to quantify 25HD using either a flame ionization or an electron-capture detector, depending on the concentration of the analyte. To compensate for differences in volumes (mainly due to injection and elution volumes) and chromatographic conditions, 2,4-pentanedione (24PD) was used as the internal standard.

### *Chemicals*

The following chemicals were used: octyl extraction columns (3 ml) (J. T. Baker, Deventer, The Netherlands), cyclohexane (GC spectrophotometric quality, J. T. Baker), 2,5-hexanedione (Aldrich-Chemie, Steinheim, F.R.G.), 2,4-pentanedione (BDH, Poole, U.K.), O-(pentafluorobenzyl)hydroxylamine hydrochloride (Chrompack, Middelburg, The Netherlands).

### *Apparatus*

The gas chromatographs used were a Hewlett-Packard Model 5890 A gas chromatograph (Hewlett-Packard Nederland, Amstelveen, The Netherlands), fitted with a split–splitless injection port and equipped with a flame ionization detector, and a Packard 428 gas chromatograph (Packard-Becker, Delft, The Netherlands), fitted with a split injection port and equipped with an electron-capture detector. Quantification was done by measurement of peak areas with a Shimadzu CR3A integrator (Shimadzu, Kyoto, Japan).

### *Chromatography*

The column used for the determination by flame ionization detection (FID) was a CP-SIL-19 CB capillary column (25 m × 0.25 mm I.D., 0.2 $\mu$m film thickness) (Chrompack). The temperature of the column was initially 60°C for 1 min, then increased to 200°C at 30°C/min. The carrier gas was hydrogen, and the column head-pressure 80 kPa. The flow-rate of nitrogen as the make-up gas was 30 ml/min. The split flow-rate was set to 10 ml/min and was suspended for the first 30 s of the run for splitless injection (injection volume 1 $\mu$l).

The column used for the determination by electron-capture detection (ECD) was the same as that used for the FID analysis. The temperature of the column was initially 130°C for 1 min, then increased to 230°C at 12°C/min. The carrier gas was nitrogen and the column head-pressure 100 kPa. The detector make-up gas

was nitrogen at a flow-rate of 30 ml/min. The split ratio was 1:20 (injection volume 1 μl).

*Sample preparation*

Urine or serum (2.8 ml) was pipetted into 10-ml tubes fitted with a screw-cap coated with PTFE. To each sample, 100 μl of a solution of 3 mg/l 24PD were added as an internal standard. After the addition of 100 μl of the solution of 5 mg/ml O-PFBHA (prepared daily), the sample was left for at least 12 h at room temperature. The reaction products were extracted using solid-phase extraction cartridges, which were conditioned with 10 ml of methanol followed by rinsing with 15 ml of water. After conditioning of the cartridge, the sample was applied. Before elution of the analytes with cyclohexane (twice 300 μl) the cartridge was dried for 20 min under vacuum (20 mmHg).

## RESULTS

Fig. 1. shows the ECD chromatograms of a blank urine sample (A), of the same urine spiked with a standard of 25HD (B) and a urine sample from a person exposed to *n*-hexane (C). Fig. 2 shows the FID chromatogram of a urine spiked with a standard of 25HD. 25HD and 24PD give three isomers after reaction with O-PFBHA. They were well separated under the FID chromatographic conditions, but under the ECD conditions (Fig. 1), two 25HD peaks overlapped. However, because the sum of the peak areas of all isomers of 25HD oximes was used for the calculations, their separation was not relevant.

As can be seen from the Fig. 1, 25HD was present in small amounts in blank urine. The concentration of 25HD in the urine of non-exposed subjects was, however, significantly lower than its concentration after *n*-hexane exposure. In ten blank urine samples (from laboratory personnel), the mean concentration of 25HD was 6.4 μg/l (S.D. 2.6).

*Precision*

To estimate the precision of the method, two urine samples with different concentrations of 25HD were analysed eight times each. The concentrations of 25HD and corresponding relative standard deviations from the mean values are presented in Table I.

*Limit of detection*

The limit of detection, defined as twice the noise level, was 4 μg/l with ECD and 50 μg/l with FID.

*Linearity*

Calibration curves from spiked urine and serum samples were linear over concentration ranges 4–100 μg/l for ECD and 0.05–10 mg/l for FID.

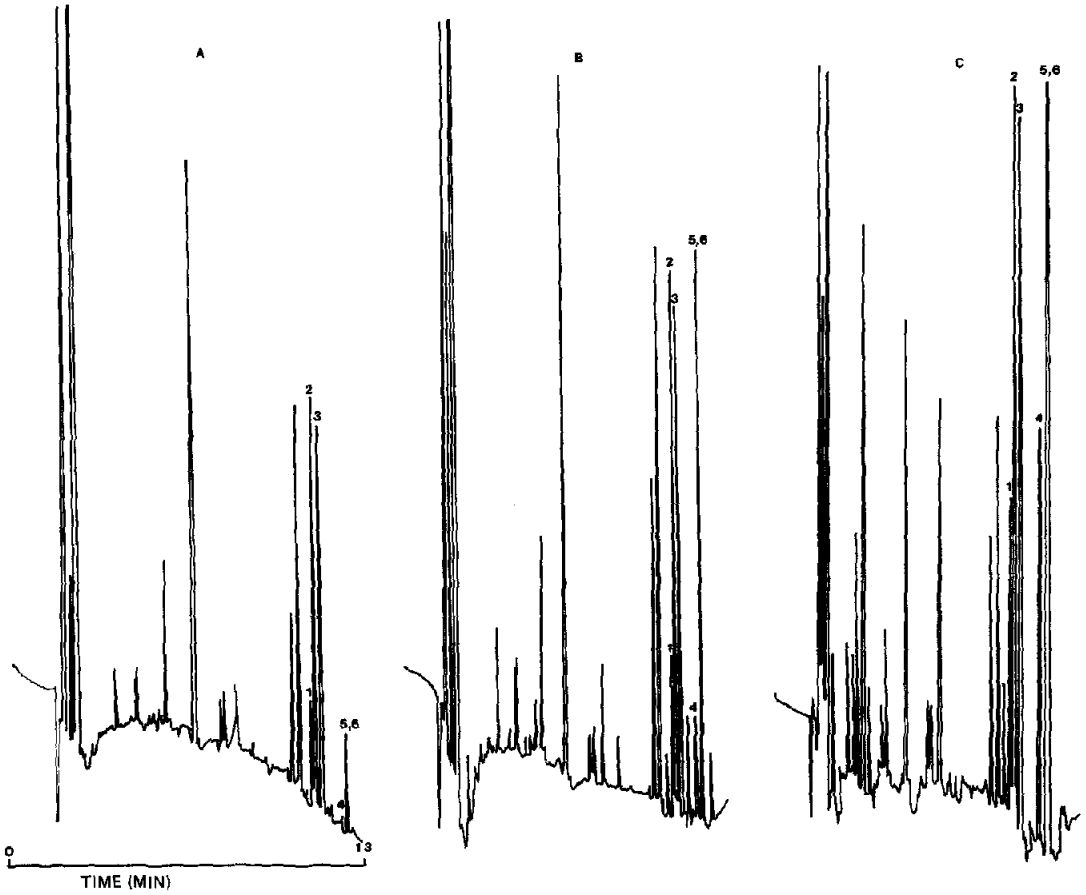


Fig. 1. ECD chromatograms of (A) blank urine samples, (B) blank urine sample spiked with a standard of 2,5-hexanedione (0.1 mg/l) and (C) a urine sample from a person exposed to 180 mg/m$^3$ *n*-hexane for 44 min. Peaks: 1, 2, 3 = isomers of O-(pentafluorobenzyl)oxime of 2,4-pentanedione (I.S.); 4, 5, 6 = isomers of O-(pentafluorobenzyl)oxime of 2,5-hexanedione.

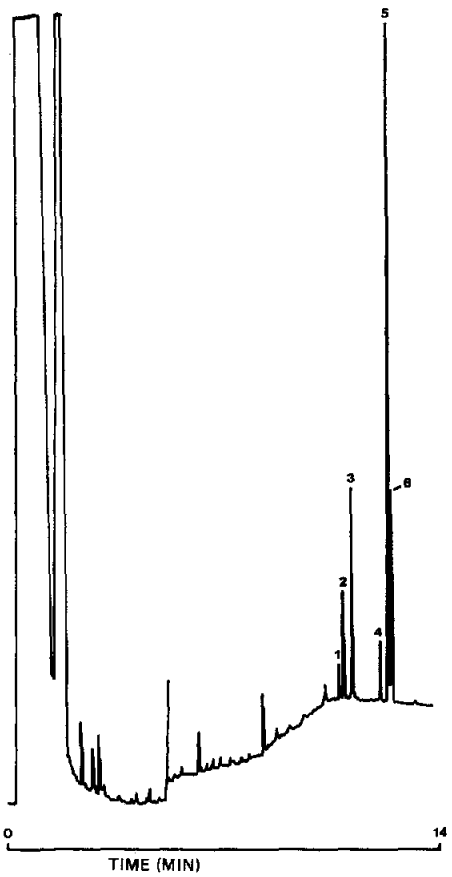


Fig. 2. FID chromatogram of urine blank spiked with a standard of 2,5-hexanedione (2 mg/l). Peaks as in Fig. 1.

TABLE I

ASSAY PRECISION

| Concentration (mg/l) | Relative standard deviation (%) ($n=8$) | |
|---|---|---|
| | ECD | FID |
| 0.052 | 3.8 | |
| 0.104 | 3.3 | |
| 0.148 | | 4.5 |
| 0.296 | | 5.0 |

## DISCUSSION

In urine, 25HD is usually determined after hot acid pretreatment in order to hydrolyse conjugated metabolites of *n*-hexane [9–13]. Recently it has been shown, however, that in such extreme circumstances, another metabolite of *n*-hexane, 45DH2H is converted into 25HD [11,12]. The increase in 25HD as a result of that conversion was ten-fold in rats exposed to *n*-hexane. Summation of these two metabolites, as suggested by some authors [11,12], is, we feel, questionable: the different toxicokinetics, and probably toxicodynamics, of these metabolites does not allow such a simplified approach. Another problem that arises is that a pure standard of 45DH2H is not available. Since there are uncertainties about the completeness of the conversion, any quantification is impossible. With the method presented here we were able to measure 25HD in serum and in urine at very low concentrations, especially when using ECD. As can be seen from Fig. 1, 25HD could easily be detected in urine of a person exposed to 180 mg/m$^3$ *n*-hexane for only 44 min. However, with high exposure to *n*-hexane, FID might be a better choice than ECD, because ECD has a limited linear range.

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