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Note

Determination of total 2,5-hexanedione by reversed-phase high-performance liquid chromatography

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Occupational exposure to *n*-hexane or related compounds has been reported to cause peripheral neuropathies which manifest themselves as leg weakness progressing to paralysis [1–3]. Toxicological studies have shown that 2,5-hexanedione is the active metabolite responsible [4–6]. 2,5-Hexanedione has also been shown to be toxic in vivo in rats [7] and in vitro for human granulocytes preparation [8] and other cell lines [9–11]. The urinary excretion of 2,5-hexanedione is used to monitor occupational exposure to *n*-hexane, because the concentration of 2,5-hexanedione in urine is closely related to the concentration of *n*-hexane in air [12,13].

Techniques based on gas chromatography with electron-capture detection [14,15] have already been described for detection of 2,5-hexanedione in urine, and a normal-phase high-performance liquid chromatography (HPLC) method has been proposed to determine the *n*-hexane metabolites [16]. The aim of our study was to evaluate an alternative method utilizing reversed-phase HPLC to measure the concentration of 2,5-hexanedione in the urine of occupationally *n*-hexane-unexposed subjects. We have developed a method based on hydrolysis of urine with hydrochloric acid (0.1 ml) for 1 h at 100°C, followed by diethyl ether extraction. Since the retention time of 2,5-hexanedione is 18 min, this method is suitable for routine biological monitoring of workers exposed to

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n-hexane Furthermore, the low level of the minimum detectable amount makes it possible to evaluate the urine concentration of 2,5-hexanedione in subjects not exposed to *n*-hexane

EXPERIMENTAL

Chemicals

2,5-Hexanedione and 1-methyl-2-pyrrolidone were from E Merck (Darmstadt, F R G), hydrochloric acid, diethyl ether, acetonitrile and bidistilled water of HPLC grade were purchased from Carlo Erba (Milan, Italy) All other chemicals were of the highest analytical grade available Filters (0 22 μm) were obtained from Millipore (Bedford, MA, U S A) Stock solutions of 2,5-hexanedione and 1-methyl-2-pyrrolidone in water (HPLC grade) were prepared and stored at -20°C for more than 2 weeks without appreciable degradation

Extraction

2,5-Hexanedione was extracted from urine using the method of Perbellini et al [14] with some modifications Acid hydrolysis of 1 ml of urine with 0 1 ml of HCl (final concentration 1 1·10^{-3} *M* HCl) was carried to estimate the total concentration of 2,5-hexanedione This was achieved by incubation in an oven at 100 C for 1 h 2,5-Hexanedione was extracted with 1 ml of diethyl ether The upper phase was aspirated, dried under a stream of nitrogen, and reconstituted in 0 5 ml of HPLC-grade water Samples were filtered through a 0 22-μm Millipore filter and analysed by reversed-phase HPLC 1 Methyl-2-pyrrolidone was used as internal standard It was added to urine at the concentration of 2 6·10^{-8} *M* before the extraction procedure

Chromatographic conditions

The HPLC apparatus consisted of two Kontron (Munich, F R G) Model 420 pumps, a 735 LC programmable variable-wavelength UV-visible detector equipped with an 8-μl flow cell, a 7125 Rheodyne injection valve and an I-459 integrator (Kontron) The separation was performed using a 5-μm particle size Supelcosil LC-18 column (25 cm×4 6 mm I D , Supelco, Bellefonte, PA, U S A) protected with a stainless-steel guard column (2 cm×4 6 mm I D) packed with 40-μm particle size Pelliguard LC-18 The injection volume was 20 μl The separation was carried out under the following conditions solvent, water–acetonitrile (95 5, v/v), flow-rate, 1 1 ml/min, detection wavelength, 233 nm At this wavelength 2,5-hexanedione showed maximum absorbance, as shown in Fig 1 1-Methyl-2-pyrrolidone also absorbs at this wavelength

To check the recovery, we added 2,5-hexanedione to urine specimens from ten healthy subjects who had not been exposed to *n*-hexane To verify the technique we performed urine analysis of ten *n*-hexane-exposed and ten normal unexposed male subjects The exposed workers used glues containing *n*-hexane

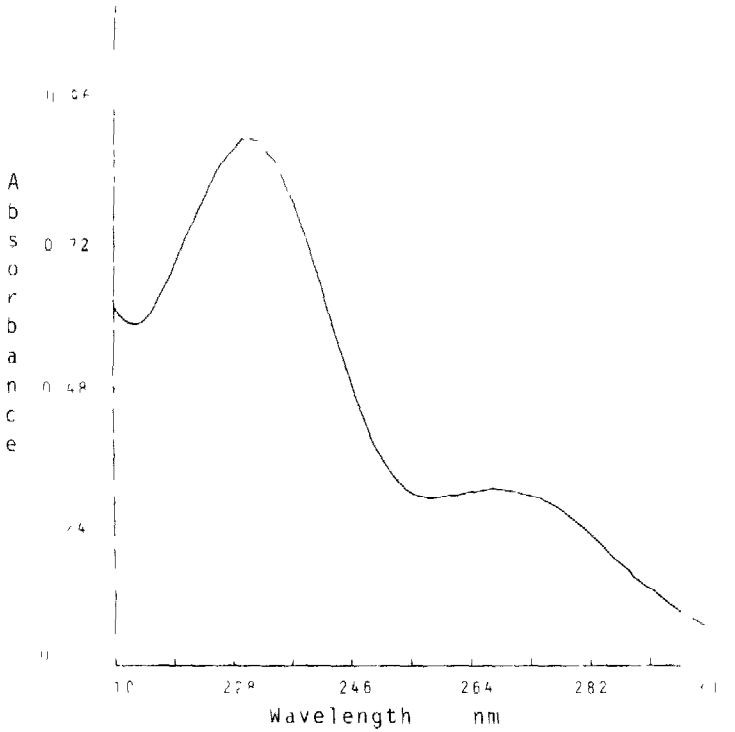

Fig 1 UV spectrum of 2,5-hexanedione in water–acetonitrile (95 5 v/v) recorded from 210 to 300 nm with a Beckman DU-50 spectrophotometer at 20°C with a scan speed of 750 nm/min

in a shoe factory All urines were from spot samples collected at the end of a weekly shift between 4 and 6 p.m They were kept for 7 10 days at −20°C until analysis All determinations were carried out in duplicate

RESULTS AND DISCUSSION

2,5-Hexanedione is the neurotoxic metabolite of *n*-hexane [4–6], which is a widely used solvent in paints, varnishes and glues, as well as in light petroleum and gasolines. In subjects exposed to *n*-hexane vapour, such as workers in shoe factories or the leather industry, 2,5-hexanedione can be present in the urine in higher concentrations than in unexposed subjects In fact a very low level of 2,5-hexanedione can also be found in subjects who are not usually in contact with *n*-hexane vapours [17], and this seems to be due to endogenous production of *n*-hexane via lipid peroxidation [15] However, the amount of 2,5-hexanedione in exposed subjects can be up to 20 times that found in normal subjects and at concentrations higher than $4\,3 \cdot 10^{-5}$ *M* which is considered by the American Conference on Hygiene of Governmental Industrial Hygienists [18] to be the threshold value beyond which 2,5-hexanedione can damage human

health Neurotoxic effects induced by 2,5-hexanedione are related to 2,5-hexanedione urinary levels [6] which, in turn, depend on the concentration of *n*-hexane in air [12,13] For this reason it is advantageous to have a simple method that allows the evaluation of this compound in urine samples

The extraction was carried out with hydrochloric acid in order to have a pH of 0 1, which is necessary to release 2,5-hexanedione conjugates [15] Increasing the pH value to 3 reduces the amount of 2,5-hexanedione released Since the acid-sensitive conjugates of 2,5-hexanedione are not known [19], we cannot rule out the possibility that they produce an artefact peak However, the recovery studies (Table I) show that under the experimental conditions described the recovery of 2,5-hexanedione ranges from 84% to 88% The internal standard has a recovery value of 88% This experiment was performed adding three different amounts (10, 25 and 50 pmol) of 2,5-hexanedione to urine samples from normal subjects whose 2,5-hexanedione concentration had been previously measured, and it confirmed the validity of the extraction procedure

The reversed-phase HPLC procedure is simple and makes it possible to detect this metabolite down to a detection limit of 1 pmol, at a signal-to-noise ratio of 3 1, which corresponds to the injection of 20 μl of a solution containing 50 nmol/l of 2,5-hexanedione

Fig 2A shows the HPLC separation of 2,5-hexanedione and of 1-methyl-2-pyrrolidone Under the chromatographic conditions described under Experimental, the two peaks appeared, respectively, at 8 5 and 18 4 min, with detection at 233 nm Fig 2B shows the chromatographic profile obtained from a urine sample of one *n*-hexane-exposed subject to which no internal standard was added Fig 2C shows the chromatographic profile of the same urine sample to which the internal standard was added. Comparison of the two profiles shows that, at ca 8 min in the sample with no 1-methyl-2-pyrrolidone, no appreciable peak is found

In Fig 2C and D two chromatograms are compared, one from an *n*-hexane-exposed (C) and one from an unexposed (D) subject under the same experi-

TABLE I

RECOVERY FROM HUMAN URINE

The urine samples used contained 1 7 10^{-6} ±0 1 10^{-1} *M* 2,5-hexanedione, which was the mean value obtained by extracting 3 times separately the same urine sample

Amount added[a] (pmol)	Amount recovered (pmol)	Recovery (%)
10 0	8 6	86
25 0	21 0	84
50 0	44 0	88

[a]For this experiment we used urine samples from one normal subject

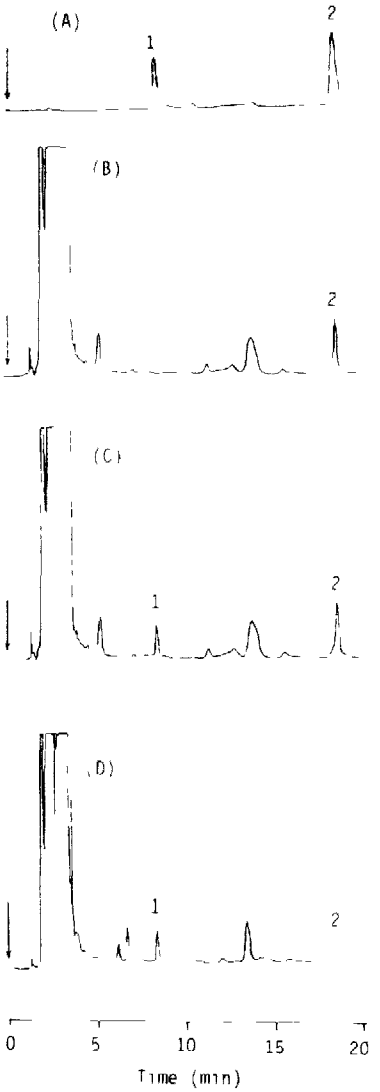

Fig 2 Reversed-phase HPLC determination of 2,5-hexanedione in water (A) and in urine (B C, D) In A the 2,5-hexanedione concentration was 8 7 10^{-6} *M* Chromatograms C and D show the separation of 2,5-hexanedione in urine from *n*-hexane-exposed and unexposed workers respectively 1 Methyl-2-pyrrolidone added as internal standard, elutes after 8 min Chromatogram B shows the chromatographic profile of the same urine as C, but without 1 methyl 2 pyrrolidone comparison shows that at ca 8 min in B there is no appreciable peak Peaks 1 = 1 methyl-2-pyrrolidone, 2 = 2,5-hexanedione Detection at 233 nm, 0 1 a u f s

mental conditions. This indicates that the detection limit of the present method makes it possible to detect the presence of 2,5-hexanedione in urine samples from unexposed subjects, who usually show a 2,5-hexanedione concentration

TABLE II

REPRODUCIBILITY OF THE DETERMINATION IN URINE SAMPLES OF EXPOSED SUBJECTS

Subject	Concentration (μmol/l)	Coefficient of variation[a] (%)	
		Intra-assay	Inter-assay
1	21 9	11 2	13 3
2	43 8	4 9	8 3
3	87 6	4 1	4 8

[a]Calculated on the basis of ten determinations for each subject

ranging from $1\,3 \cdot 10^{-6}$ to $5\,2 \cdot 10^{-6}$ *M* [10,11]

Concerning the possibility of interferences due to the presence of other *n*-hexane metabolites, it should be pointed out that the level of 2,5-hexanedione is much higher than the levels of 2-hexanol and γ-valerolactone the other metabolites of *n*-hexane These two compounds also absorb at a different wavelength significantly lower than 233 nm Therefore it is reasonable to expect no significant interference from either compound

Results of replication of the recovery concentration curve over three concentrations show a mean standard curve which, at concentrations lower than $5 \cdot 10^{-8}$ *M*, fits a linear equation ($y = 0\,11x - 0\,009$, $r = 0\,99$, $n = 6$) The results obtained from our laboratory in exposed workers from a shoe factory ranged from 12 3 10^{-6} *M* to $42\,9 \cdot 10^{-6}$ *M* with a mean ± S D of $24\,6 \pm 11\,2 \cdot 10^{-6}$ *M* in urine In unexposed subjects the concentration of 2,5-hexanedione ranged from $1\,3 \cdot 10^{-6}$ *M* to $5\,2 \cdot 10^{-6}$ *M* with a mean ± S D of $3\,0 \pm 1\,3 \cdot 10^{-6}$ *M* No differences were observed between male and female subjects The reproducibility studies were carried out using the urine samples of three exposed subjects on the same day of urine collection and two days after the collection, as reported in Table II The low coefficients of variation indicate that the procedure is reproducible and reliable

CONCLUSION

This paper describes a reversed-phase HPLC method for the rapid separation of 2,5-hexanedione (18 min) This method is particularly useful for evaluating this compound in urine samples of workers exposed to *n* hexane The procedure is simple, reproducible and reliable, making the method suitable for routine analysis of this metabolite in urine

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