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High-performance liquid chromatographic determination of urinary 2,5-hexanedione as mono-2,4-dinitrophenylhydrazone using ultraviolet detection

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Abstract

The good correlation between exposure to *n*-hexane and 2,5-hexanedione urinary excretion confers on this diketone an important toxicological meaning. This paper proposes a reversed-phase HPLC method which includes, after acid hydrolysis, a derivatization step of 2,5-hexanedione with 2,4-dinitrophenylhydrazine at 70°C for 20 min. The reaction conditions, such as temperature, reagent concentration and time, are optimized so as to allow the condensation of a single carbonyl group. A linear response was obtained in the 0.19–20.0 mg/l range with a detection limit of 0.03 mg/l, corresponding to a signal-to-noise ratio of 3. A phosphate buffer (pH 3.3)–acetonitrile mixture (50:50) as the eluent and UV detection at 334 nm were used.

1. Introduction

n-Hexane, a solvent widely used in the shoe manufacturing and adhesive production industries, is well known to be neurotoxic. The dangerous species is 2,5-hexanedione (2,5-HD) and for this reason is the most frequently studied *n*-hexane urinary metabolite [1–4]. Although this diketone also occurs at low concentrations (0.1–0.68 mg/l) in the urine of unexposed people [5], toxicological studies have demonstrated good correlation between exposure of *n*-hexane in workplaces and 2,5-HD urinary excretion [6]. The current threshold value proposed by ACGIH [7] as biological exposure index (BEI) is 5 mg/g creatinine at the end of a workshift. 2,5-HD determination usually involves gas chromatographic methods [8–10], the signal being detected by flame ionization detection or MS. Analysis is preceded by acid hydrolysis by which, at pH of ca. 0.5, the real metabolite of *n*-hexane (4,5-dihydroxy-2-hexanone) [11] is completely converted to 2,5-HD. The drawbacks of these methods are the time-consuming steps, severe sample manipulation (liquid–liquid or solid-phase extraction) and enrichment steps (e.g. solvent drying) to reach adequate sensitivities. Moreover, the chromatographic separation of the analyte may not be

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achieved owing to the presence of interfering compounds (e.g. 2-acetylfuran) [12]. Increased sensitivity has been obtained by derivatizing 2,5-HD, using O-(pentafluorobenzyl)hydroxylamine followed by solid-phase extraction and electron-capture detection (ECD) [13].

Many sample preparation problems, also improving sensitivity, may be solved by an HPLC method. Theoretically, direct UV detection of 2,5-HD must be ruled out, due to its negligible value of molar absorptivity [14]. This aspect has already been emphasized [15,16] so much so that the presence of an impurity in commercial 2,5-HD had misled some authors in its direct HPLC–UV determination [17,18]. In our opinion, the right way to deal with HPLC analysis of this diketone must involve a derivatization step. Various ketones, such as acetone or 2-butanone, present in biological fluids, have been derivatized to 2,4-dinitrophenylhydrazone, a classical derivative of the carbonyl group [19,20]. In typical reaction conditions, 2,5-HD also gives hydrazone, but identified as a bis-derivative. Although this compound of high molecular mass does have some qualitative interest, it becomes useless for HPLC determinations due to its strong interaction with the reversed-phase columns and low solubility in the common chromatographic eluents (water, methanol and acetonitrile). Pursuing our interest in 2,4-dinitrophenylhydrazine (2,4-DNPH) as a reagent for 2,5-HD, the derivatization conditions have been modified and optimized so as to yield the mono-derivative as the main reaction product. This chemically stable compound shows good HPLC behaviour, such as reasonable retention time and significant UV response. This work thus proposes a simple and sensitive HPLC method for the determination of 2,5-HD as a mono-hydrazone in the urine both of workers exposed to *n*-hexane and of unexposed people.

2. Experimental

2.1. Materials and reagents

2,4-DNPH was purchased from Merck (Darmstadt, Germany). 2,5-HD, KH_2PO_4, sodium octanesulphonate, acetonitrile (ACN) and other HPLC-grade solvents were supplied by Sigma–Aldrich (Milan, Italy). Water was produced by a MilliQ-Plus apparatus. Disposable 0.2-μm Millex-GS filters were furnished by Millipore (Milan, Italy). A 0.2% (w/v) aqueous solution of 2,4-DNPH in 2 *M* hydrochloric acid was prepared daily.

2.2. Identification of 2,5-HD hydrazones and MS instrumentation

Mass spectrometric identification of the bis-derivative was carried out using a VG-ZAB-2F Instrument (Altrincham, UK) operating in EI mode (70 eV, 200 μA, source temperature 200°C). Samples were introduced directly in the ion source by means of a probe heated to 280°C. The compound was synthesized according to the literature procedure [21].

MS identification and confirmation of the mono-derivative was carried out using crude reaction solution analysed by a SCIEX API III (Perkin Elmer, Norwalk, CT, USA) triple quadrupole mass spectrometer equipped with ionspray source (orifice voltage 65 V) and coupled to the HPLC chromatographic system, because of the difficulty of insulating a solid, pure sample from the reaction mixture. The analytical separation was performed by a C_{18} column (33 × 4.6 mm I.D., 3-μm particles) at a flow-rate of 1 ml/min of acetonitrile–water (35:65). Before entering the mass spectrometer, the flow was split 1:25 in order to feed the ionspray source with 40 μl/min of eluate.

The compound was synthesized according to our experimental conditions, starting from a 2,5-HD aqueous solution with hydrazine reagent and acetonitrile added.

2.3. Preparation of urine samples

Urine samples (5 ml) from persons exposed or unexposed to *n*-hexane and 1 ml of concentrated hydrochloric acid (35%, v/v) were pipetted into

a screw-capped vial, mixed and then placed for 40 min in an oven at 100°C for acid hydrolysis. After cooling to room temperature, the reaction mixture was filtered through a 0.2-μm disposable filter. The filtrate (1 ml) was mixed with 1 ml 2,4-DNPH reagent solution and 1 ml of HPLC-grade acetonitrile in a screw-capped vial and then heated for 20 min in an oven at 70°C. After cooling, 50 μl of the solution were injected into the HPLC apparatus.

The calibration curve in the 0.19–20 mg/l range was obtained by standard additions of 2,5-HD to hydrolyzed urine of unexposed people. A concentrated aqueous solution of 2,5-HD (390 mg/l) was prepared by dissolving 100 μl of diketone in 250 ml of HPLC grade water. Successive dilutions allowed to perform additions of low volumes (10–100 μl) of standard to urine so as to obtain the target concentrations.

2.4. HPLC instrumentation and conditions

All the HPLC measurements of 2,5-HD derivative were performed by a Model 410 pump, an LC-95 UV–Vis variable wavelength detector, an LC-600 autosampler and a CC-12 integrator, all from Perkin Elmer (Norwalk, CT, USA). Using the diode array as a detector, pump Model 200, detector LC 235C and autosampler IS 200, all from Perkin Elmer, have been used together with an Epson 486 DX computer (Turbochrome as the software) for acquisition and processing of data.

Two different separation columns were used: (a) column 6-ODS-8-5 SGE (150 × 4.6 mm, 5 μm), mobile phase (20 m*M* KH_2PO_4 and 1 m*M* sodium octanesulphonate buffer solution, adjusted to pH 3.3 with H_3PO_4)–acetonitrile (50:50) at a flow-rate of 1 ml/min, used for routine analyses; (b) Supelcosyl LC-18 column (250 × 4.6 mm; 5 μm), mobile phase acetonitrile–water (40:60) for 28 min, followed by a linear gradient (in 5 min) up to 100% acetonitrile, maintained for 15 min at a flow-rate of 1.2 ml/min, also proposed for bis-derivative elution. The detection wavelength in both cases was 334 nm.

3. Results and discussion

The application of 2,4-DNPH with carbonyl compounds under the usual condensation conditions led, in the case of 2,5-HD, to the formation of the bis-derivative. This yellow solid, separated from the reaction mixture, was confirmed in direct MS analysis to have a molecular peak at *m/z* 474. Nevertheless, this compound showed low solubility in reversed-phase HPLC eluents such as water, methanol and acetonitrile, and an absorbance maximum in chloroform solution at 366 nm [21]. Under HPLC analytical conditions, the above characteristics led to long retention times while the high 2,4-DNPH concentrations (2.5–5%) proposed in the literature [20, 22], led to undesired formation of precipitate and high background levels in the blanks. Moreover, the strongly acidic matrix could cause damage to the column and autosampler system. So these derivatization conditions and bis-derivative cannot successfully be used in reversed-phase HPLC determinations.

As 2,4-DNPH solutions at concentrations lower than those quoted above, have been proposed for the derivatization of other carbonyl compounds in urine [19], this method was tried both with aqueous solutions of 2,5-HD and with urine samples from unexposed people spiked with known amounts of 2,5-HD. The reaction conditions were as follows: 1 ml of 0.03% (w/v) 2,4-DNPH in 2 *M* HCl and 1 ml of acetonitrile were added to 1 ml of sample containing 2,5-HD. Acetonitrile was added to keep the liquid phase homogeneous and to avoid precipitate formation. The mixture was heated for 30 min at 50°C. The analytical conditions described in point (a) of Section 2.4 were used for recording the chromatograms by a diode-array detector. An unknown compound with a retention time of 18.6 min has been detected, having λ_{max} at 334 nm and a linear response in the range 0.19–20 mg/l of 2,5-HD. As an example, the chromatogram of a urine sample spiked with 2.3 mg/l of 2,5-HD is shown in Fig. 1. Under these conditions, the bis-derivative was not eluted.

Using aqueous solutions of 2,5-HD and modified eluent conditions, as reported in point (b) of

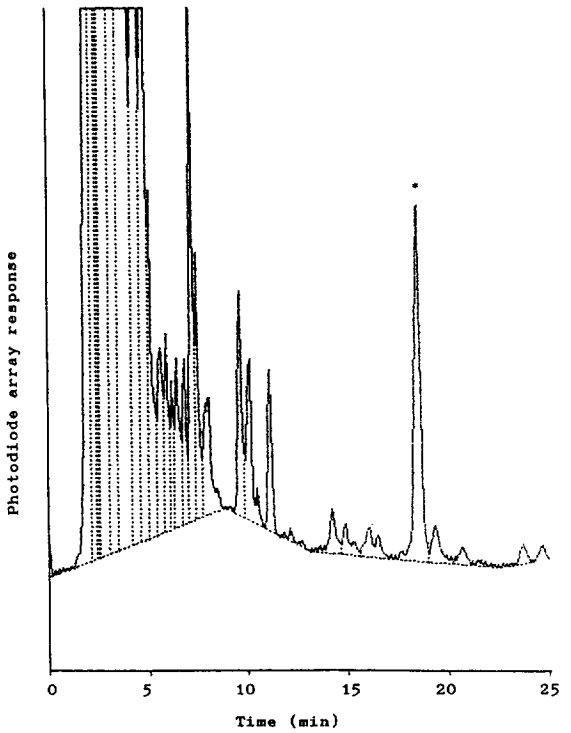

Fig. 1. HPLC–photodiode-array chromatogram (334 nm) of 2,5-HD mono-hydrazone (peak labelled with asterisk at 18.6 min) obtained from urine spiked at a concentration of 2.3 mg/l 2,5-HD, eluted according to conditions (a) in Section 2.4.

Section 2.4, the presence of two separate compounds was observed. The chromatogram of an aqueous solution containing 7.8 mg/l of 2,5-HD is shown in Fig. 2. Peak 1 is that of the principal, unknown condensation compound; peak 2 was identified as that of the bis-derivative. Peak 1 was identified by analysing the above reaction solution by the SCIEX API III quadrupole coupled with an HPLC separation system. The compound was unequivocally identified as mono-2,4-dinitrophenylhydrazone, with protonated molecular ions at m/z 295 (Fig. 3).

From a yield point of view, the reaction favouring the mono-derivative was studied considering the main parameters, such as temperature, time of reaction, concentration of 2,4-DNPH, possible interferences and product stability.

Temperature proved to be the most important parameter. Fig. 4A shows the trend of reaction yield as a function of temperature. The best peak performance was obtained at 70°C, with an area increase of about 60% as compared to conditions at 50°C.

The effect of reaction time was also studied using spiked urines (Fig. 4B). The maximum amount of analyte was produced between 20 and 25 min from the beginning of the reaction. The best mono-derivative yield was achieved at 70°C with a reaction time of 20 min.

As noted above, as high 2,4-DNPH concentrations are not recommended, the influence of the reagent concentration over a 0.03–0.6% (w/v) specific range has been studied. Experimental data demonstrated an increased yield with a plateau near 0.2% (Fig. 4C). Values of 0.3% (w/v) and higher gave results which were not reproducible. Furthermore, greater difficulties in reagent dissolution, with possible precipitate formation during the stay in the autosampler were observed. The optimal concentration was identified at 0.2% (w/v) 2,4-DNPH.

The urine samples have not shown significant interferences owing to the good resolution of the column. Furthermore, the used wavelength (334 nm) was different from the maximum UV absorption (near 365 nm) of the most 2,4-dinitrophenylhydrazones obtained from urinary ketone bodies.

The last variable considered was the stability of the mono-derivative as a function of time, because "normal" urine contains other carbonyl compounds such as acetone, hydroxyacetone, acetoacetic, β-hydroxybutyric, α-ketoglutaric, glyoxylic and pyruvic acids. 2,4-DNPH is present in large excess with respect to the low concentration of 2,5-HD, but the presence of the above carbonyl compounds and the possibility of formation of the bis-hydrazone of 2,5-HD may theoretically influence condensation of the mono-derivative. The stability test was performed using a urine pool from unexposed people (5 replicates) spiked with the same amount of 2,5-HD (9.75 mg/l). All the urine samples, subdivided in 5 aliquots, were treated as reported in Section 2 and sequentially injected, prolonging

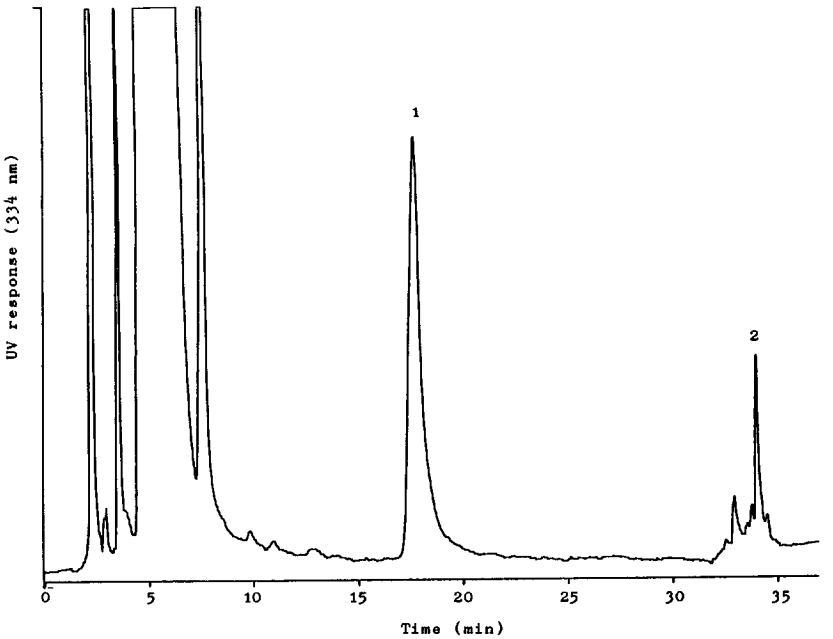

Fig. 2. HPLC chromatogram of 2,5-HD hydrazone obtained from a 2,5-HD aqueous solution containing 7.8 mg/l diketone, eluted according to conditions (b) in Section 2.4. Peaks 1 and 2: mono- and bis-derivative, respectively.

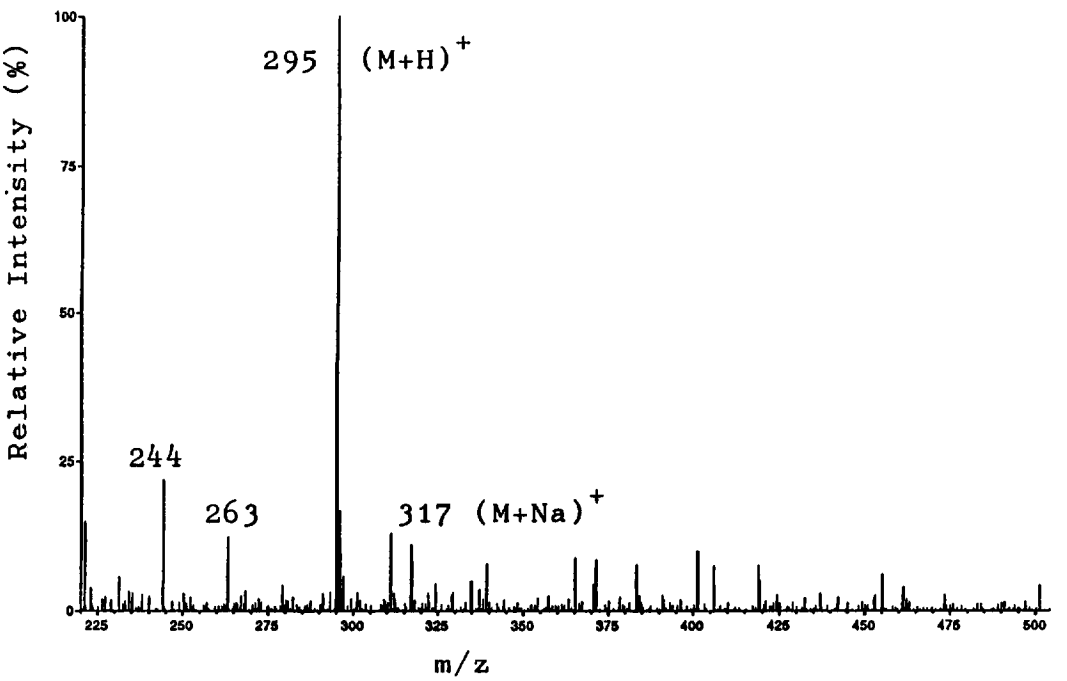

Fig. 3. Mass spectrum of 2,5-HD mono-hydrazone as obtained from analysis by the HPLC–SCIEX API III system.

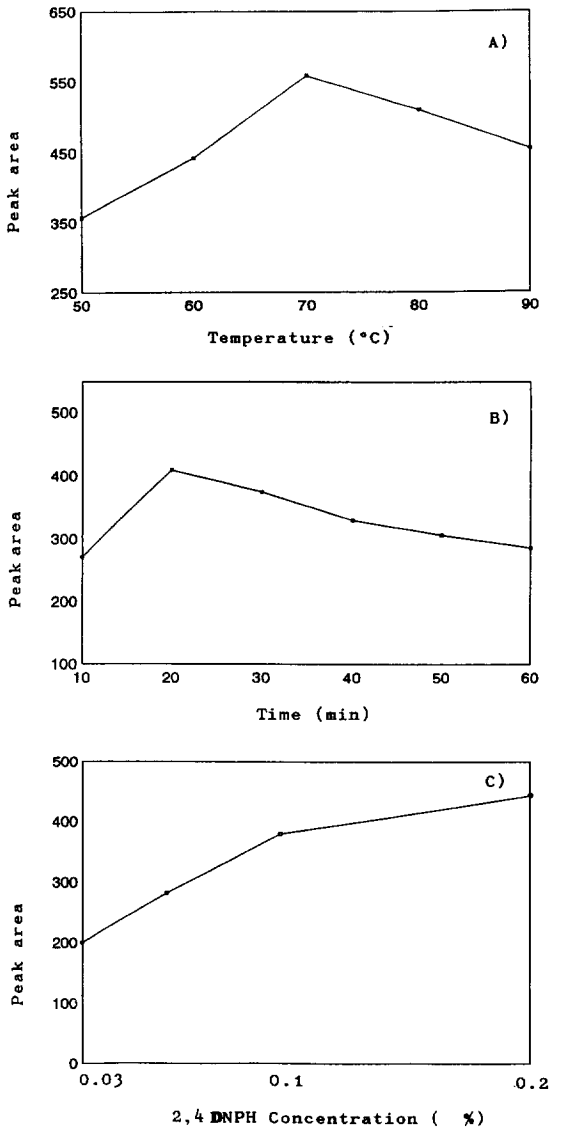

Fig. 4. Peak area of mono-derivative from urine spiked with 7.8 mg/l 2,5-HD according to (A) temperature, (B) time and (C) 2,4-DNPH concentration. In the cases (A) and (B) 0.1% 2,4-DNPH reagent solution was used. A temperature of 70°C was chosen in experiments (B) and (C).

analysis times over a total period of about 12 h. Results are shown in Fig. 5, where the peak areas of the 2,5-HD derivative are plotted against time. Each point represents the average value ($n = 5$) calculated at intervals of about 3 h. All the data

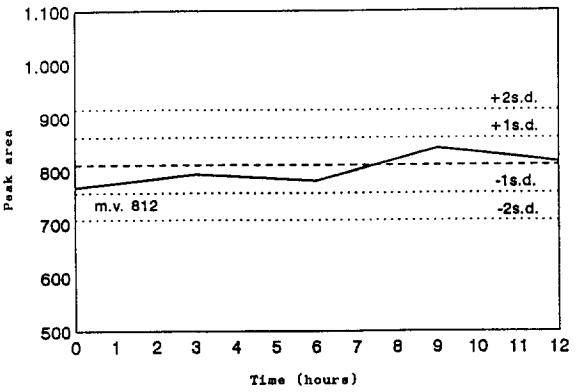

Fig. 5. Mono-derivative peak average area obtained by measuring the same 5 urine samples spiked with 9.75 mg/l 2,5-HD every 3 h.

fall within a narrow range with respect to average values.

This method did not require any particular maintenance of the HPLC system because, in the testing conditions, no problem of precipitate formation or damage to the autosampler or analytical column was observed. Nevertheless, every sample injection was followed by careful washing of needle and loop with a solution of acetonitrile–water (50:50). At the end of every analytical session, the whole system was carefully washed with 100% acetonitrile, so as to elute the derivatives of possible heavier compounds present in the urine samples.

Performance of the method and data quality control

The present analytical method allows analysis both of urine of workers professionally exposed to *n*-hexane and of unexposed people. Fig. 6 shows the chromatograms of urine from unexposed subjects (A), spiked urine (B), and urine from exposed workers (C). The method ensured a detection limit of 0.03 mg/l (signal-to-noise ratio = 3) and a good linear regression equation in the 0.19–20 mg/l range ($y = 0.92x - 0.008$; $r^2 = 0.998$, $n = 8$, where x is 2,5-HD concentration and y is UV-detector area counts). The total yield of the method in urine with respect to

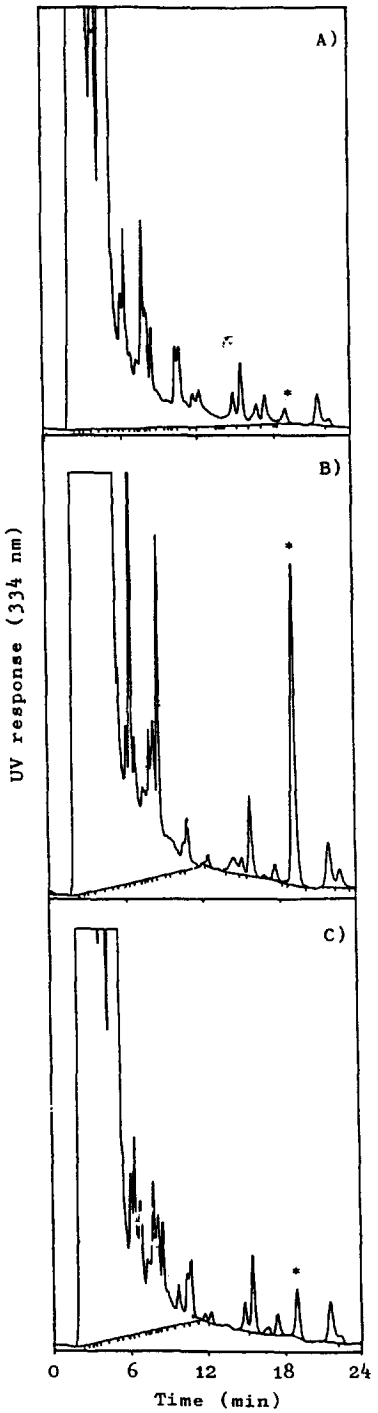

Fig. 6. HPLC–UV chromatogram of 2,5-HD mono-hydrazone (peak labelled with asterisk) obtained from: (A) urine of unexposed persons (0.35 mg/l); (B) urine spiked with 5.85 mg/l 2,5-HD; and (C) urine of exposed workers (1.20 mg/l), eluted according to condition (a) in Section 2.4.

Table 1
Comparison between data obtained from inter-laboratory measurements concerning 2,5-HD (mg/l) determination in real urine samples

	Level		
	Low	Medium	High
Our mean	0.29	1.24	3.70
Our C.V. (%)	8.7	5.2	4.0
General mean	0.35	1.22	3.56
General C.V. (%)	9.1	5.5	5.4

corresponding aqueous solutions was about 94%, using the above concentrations. The standard deviation of 2,5-HD recovery from spiked urine of five different unexposed persons was about ±1% on condition that the samples were fresh.

The method was tested by inter-laboratory controls. Three samples of real urine corresponding to three exposure levels of urinary 2,5-HD were analysed by nine public laboratories, six of which performed analysis with gas chromatography and three with this proposed HPLC method. The concentrations defined "low" corresponded to values lower than 0.5 mg/l, usually found in urine of unexposed people; while "medium" (0.5–2.5 mg/l) and "high" (2.5–5 mg/l) levels were referred to current ACGIH BEI in urine of professionally exposed workers. Each laboratory analysed the same sample seven times. The results are shown in Table 1.

4. Conclusions

The proposed HPLC procedure for the 2,5-HD determination shows good efficiency in terms of sensitivity and reproducibility. Sample preparation is easy and the analytical measurements only require simple instrumentation used by all chemical-toxicological laboratories. Therefore, this method may be useful for routine analyses in laboratories specializing in biological monitoring of workers professionally exposed to *n*-hexane.

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