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Determination of 2,5-hexanedione, a metabolite of *n*-hexane, in urine: evaluation and application of three analytical methods

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Abstract

Three methods for the determination of 2,5-hexanedione (2,5-HD) in urine were compared in order to assess their applicability for toxicokinetic studies and biological monitoring of occupational exposure to *n*-hexane. Two of them were based on derivatization, followed by gas chromatography and electron-capture detection. Of these two, one is a modification of the other, already published, method. The third one involves direct extraction of 2,5-HD followed by gas chromatography and flame-ionization detection. To determine 2,5-HD in urine of workers occupationally exposed to *n*-hexane, the most straightforward method, direct extraction of 2,5-HD from urine, has been proven to be the most suitable. However, in case of very low concentrations of 2,5-HD in urine, or analysis of small samples of blood, e.g. in kinetic studies, it is necessary to use a more sensitive procedure. The sensitivity of the methods based on the derivatization of 2,5-HD followed by electron-capture detection, was, as expected, much higher in terms of analytical reliability. By using these methods, however, precautions are necessary to avoid a matrix effect.

1. Introduction

From a toxicological point of view, 2,5-hexanedione (2,5-HD) is the most relevant metabolite of *n*-hexane and methyl-*n*-butylketone, as it is generally assumed to cause peripheral neuropathy in subjects exposed to these solvents. Several authors studied the use of 2,5-HD as a biological marker of occupational exposure to *n*-hexane and found a good correlation between environmental concentrations of *n*-hexane and the amount of 2,5-HD excreted in urine [1,2].

In most studies, the determination of 2,5-HD

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is based on the method described by Perbellini et al. [3], which involves extraction of 2,5-HD after acid hydrolysis of urine. However, Fedtke and Bolt [4,5] reported that during acid treatment, in vitro conversion occurred of another hexane metabolite into 2,5-HD. This metabolite was identified as 4,5-dihydroxy-2-hexanone and was present in urine of rats and humans after exposure to *n*-hexane. In that study, the amount of 4,5-dihydroxy-2-hexanone excreted in urine after *n*-hexane exposure was about ten times higher than that of 2,5-HD. Similar findings were reported by Perbellini et al. [6] who found a ratio between free (true) and total 2,5-HD (without and with acid hydrolysis, respectively) of about 0.08 in the urine of workers exposed to *n*-hexane. In blood this ratio was 0.5. When using urinary 2,5-HD concentrations obtained after acid hydrolysis, it has to be kept in mind that the greater part is not 2,5-HD which is formed by metabolism of *n*-hexane, but also derived from the in vitro hydrolysis of other compounds (e.g. 4,5-dihydroxy-2-hexanone). Therefore, a method in which only 2,5-HD derived from *n*-hexane metabolism is determined is to be preferred. As acid hydrolysis is a procedure used to cleave excreted conjugates, it can be omitted in the determination of 2,5-HD, as this diketone is not conjugated at all. Recently, we performed a kinetic study in which volunteers were experimentally exposed during 15 min to low concentrations of *n*-hexane [60 ppm (211 mg/m^3)] (Van Engelen et al. in preparation). To be able to detect low quantities of 2,5-HD in a small volume of blood (or urine) a very sensitive method was needed. Derivatization of 2,5-HD enables the introduction of F-atoms which leads to the possibility to use the very sensitive electron-capture detection. To reduce the costs and effort of the analysis, we investigated the suitability of liquid–liquid extraction instead of the solid-phase extraction used in the original method [7]. The third evaluated method [1] was a straightforward one, as it involves no derivatization. All three methods were, besides sensitivity, evaluated on the basis of analytical reliability: accuracy and precision.

2. Experimental

2.1. Chemicals

The following chemicals were used: 2,5-hexanedione (Aldrich Chemie, Steinheim, Germany); cyclohexane, dichloromethane, methanol, tetrachloroethylene, 3-ml (200 mg) octyl extraction columns (J.T. Baker, Deventer, Netherlands); O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride, cyclohexanone, 2,4-pentanedione (Sigma, St. Louis, MO, USA); citric acid (citrate buffer 0.34 *M*, pH 2.2), sodium chloride, hydrochloric acid (Merck, Darmstadt, Germany).

2.2. Urine samples

Urine samples were collected from ten volunteers with no known exposure to *n*-hexane or methyl-*n*-butylketone. Urine samples varied in specific density from 1.005 to 1.029 g/l and in pH from 5.06 to 7.78. Pooled blank urine was prepared by mixing these samples. The concentration of 2,5-HD in this pool was lower than the limit of detection of the methods.

2.3. Derivatization of 2,5-hexanedione followed by solid-phase extraction (method 1)

Method 1 was described by Kežić and Monster [7]. The method is based on a derivatization reaction of 2,5-HD with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), followed by a solid-phase extraction (SPE) and GC with electron-capture detection (ECD). As reported, the derivatization reaction proceeds readily in weakly acidic media (pH 4–6). Since some of our samples were above this range after addition of PFBHA, acidification with HCl was necessary.

Sample preparation

A 280-μl volume of a 5.12 μmol/l solution of 2,4-pentanedione (2,4-PD) (as internal standard) and 50 μl PFBHA (80 mmol/l) were added to 2.8 ml of urine and adjusted with 0.1 *M* HCl to

pH 4–6. Samples were subsequently rotated (10 rpm) for at least 16 h. The sample was applied to C_8 SPE columns and eluted as described by Kežić and Monster [7]. Calibration curves were prepared by adding a known amount of 2,5-HD to pooled blank urine. At least 6 different concentrations were each analyzed twice. The concentration range of the standards (0–1 μmol/l) was within the linear range of the ECD. Urine samples with a concentration above this range were diluted 10 times with pooled blank urine. To construct a calibration line, the peak heights of 2,5-HD divided by the peak height of the internal standard were plotted as a function of the 2,5-HD concentrations. For the quantitation of both 2,5-HD and 2,4-PD the peak height of the most abundant isomer was used, as the relative ratio of the three isomers of both 2,5-HD and 2,4-PD was constant.

2.4. Derivatization of 2,5-hexanedione followed by liquid–liquid extraction (method 2)

Method 2 was recently developed by us. It is based on method 1, but the SPE is replaced by a liquid–liquid extraction. Citrate buffer was added to maintain a pH of 4–6.

Sample preparation

To each 500-μl urine sample, 200 μl of citrate buffer pH 2.2 was added, as well as 50 μl of a solution of 5.12 μmol/l of 2,4-PD as internal standard and 50 μl of PFBHA solution (80 mmol/l). Samples rotated overnight for at least 16 h (10 rpm). After addition of 300 μl of cyclohexane, samples rotated for 90 min at 70 rpm. A 1-μl aliquot of the cyclohexane extract was injected onto the GC system. Calibration lines were constructed as described for method 1. Samples with a 2,5-HD concentration above 1 μmol/l were diluted 10 times with pooled blank urine.

A characteristic part of the chromatogram obtained with urine from a volunteer exposed to *n*-hexane is presented in Fig. 1.

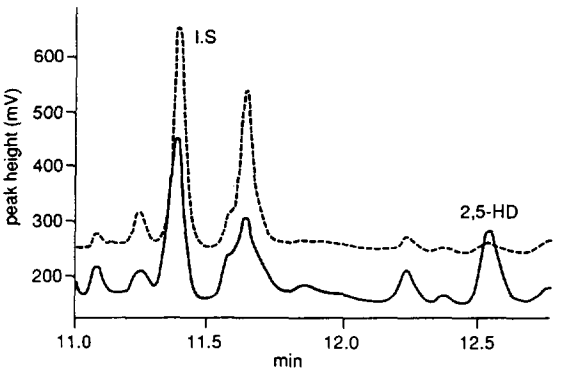

Fig 1. Part of a chromatogram of urine obtained from a volunteer experimentally exposed to *n*-hexane (56 ppm during 15.5 min) analysed according to method 2. The dotted line represents blank urine, the solid line represents urine obtained after exposure. The concentration of 2,5-hexanedione in this sample was calculated to be 0.36 μ*M*.

2.5. Direct liquid–liquid extraction (method 3)

Method 3 was described by Kawai et al. [1] and we have introduced a slight modification. The original method involves liquid–liquid extraction of 2,5-HD (without derivatization) followed by gas chromatography with flame-ionization detection (FID). To improve extraction, the urine was salted out by adding 1.5 g NaCl.

Sample preparation

To 5 ml of urine, 200 μl of a 190 μ*M* cyclohexanone solution in water was added as internal standard. After addition of 1 ml of dichloromethane, samples rotated for 15 min (70 rpm) and were subsequently centrifuged for 10 min at 2000 *g*.

A 1-μl aliquot of the dichloromethane extract was injected onto the GC system. Calibration lines were constructed as described in method 1.

2.6. Conditions

Methods 1 and 2

The GC used was a Hewlett-Packard Model 5890A equipped with an ECD and a CP-SIL-8-CB column (25 m × 0.25 mm I.D., film thickness 0.25 μm; Chrompack). Injector temperature was

set at 200°C, detector temperature at 300°C. The oven was programmed from 60°C to 90°C at a rate of 15°/min. After 2 min, temperature was increased with 15°C/min to 150°C and with a rate of 50°C/min to 200°C. Temperature was kept at 200°C for 10 min. The carrier gas was hydrogen at a flow-rate of 5 ml/min. The detector make-up gas was nitrogen at a flow-rate of 35 ml/min. The column head-pressure was 125 kPa, as measured at the initial temperature. The split flow-rate was set to 5 ml/min and was suspended for the first 20 s of the run for splitless injection (injection volume 1 μl).

Method 3

The GC used was a Hewlett-Packard Model 5890 A, equipped with a FID and a CP-SIL-43-CB column (10 m × 0.25 mm I.D., film thickness 0.20 μm; Chrompack). Injector temperature was 200°C, detector temperature 250°C. Oven temperature was kept at 50°C for 1 min, increased with 30°C/min to 120°C and subsequently to 180°C with a rate of 60°C/min. Temperature was kept at 180°C for 1 min. Split ratio was 1:10 (injection volume, 1 μl). Carrier gas was nitrogen at a rate of 1.8 ml/min and the column head-pressure was 70 kPa, as measured at the initial temperature.

2.7. Precision

A sample of pooled urine was spiked with two different concentrations of 2,5-HD: the final concentrations for method 1 and 2 were 0.2 and 0.9 μmol/l and for method 3 they were 2.0 and 8.6 μmol/l urine. Spiked urines were distributed in 10 portions which were analyzed by each method. Precision was assessed based on the coefficient of variation (C.V.) of the mean value of the 10 determinations.

2.8. Accuracy

In order to assess the accuracy of each method, we have determined recovery, the matrix effect and the bias from the true value.

Recovery

The overall recovery in the methods 1 and 2 could not be determined, as the derivatized compound was not commercially available. Therefore, the recovery of elution (method 1) or extraction (method 2) was determined. A sample of pooled urine was spiked with two different concentrations of 2,5-HD: the final concentrations for method 1 and 2 were 0.2 and 0.9 μmol/l and for method 3 were 2.0 and 8.6 μmol/l urine.

Method 1: the amount of 2,5-HD was determined in both eluents collected after two consecutive elutions. To compensate for differences in volumes, tetrachloroethylene was added to each eluate as an internal standard. Recovery of each elution is expressed as a percentage of the total amount of eluted 2,5-HD.

Method 2: urine samples after the derivatization step were extracted two consecutive times with cyclohexane containing tetrachloroethylene as an internal standard. Extraction recovery of both extractions is expressed as a percentage of the total amount of 2,5-HD extracted.

Method 3: the results of spiked urine samples were compared with those of calibration standards added directly to dichloromethane which were taken as 100% recovery.

Matrix effect/standard addition method

To examine a possible matrix effect the standard addition method was applied. Ten urines from non-exposed persons were spiked in duplicate at two concentration levels (method 1: 0.30 and 0.59 μmol 2,5-HD/l urine, method 2: 0.33 and 0.66 μmol 2,5-HD/l urine and method 3: 3.31 and 6.62 μmol 2,5-HD/l urine). For all three methods, linear regression lines were constructed from the blank value plus two standards, in duplicate. The variation in the slopes of the linear regression lines is considered to be an indicator of the matrix effect.

Bias from the true value

To assess the bias from the true (in this case the added) value, 10 urines from non-exposed persons were spiked, in duplicate, with 2,5-HD in the concentration range 0.1–10 μmol/l and

determined by all three methods. For methods 1 and 2 urine samples with a concentration above 1 μmol/l were diluted 10 times with pooled blank urine. Quantitation was done by using the calibration curve made in pooled urine. The obtained values were plotted against the added concentrations. For each method, a linear regression line was constructed and the r^2 as well as the slope were a measure for the bias from the true value.

2.9. Limit of quantitation

As lower limit of quantitation (LLQ), we used the following definition: lower limit of quantitation is that concentration of the analyte in the matrix of interest for which the 95% confidence interval does not overlap with the confidence interval of the matrix blank standard. For all three methods, a calibration line was constructed in pooled blank urine (range: 0–0.4 μmol/l for methods 1 and 2; range 0–1.6 μmol/l for method 3). The 95% confidence level was calculated, and the LLQ was determined.

3. Results

3.1. Precision

The coefficient of variation (C.V.) of the 10 determinations of the concentration of 2,5-HD in pooled blank urine, spiked at two concentration levels, is presented in Table 1. These C.V.s were less than 10% for all three methods.

3.2. Accuracy

Recovery

At the low concentration level for method 1, of the total amount of 2,5-HD eluted, 91.9% was eluted after the first elution and only 8.1% after a consecutive elution. For method 2, these percentages were 93.5% and 6.5%, respectively. At the higher concentration level, these percentages were 90.7% and 9.3% for method 1 and 94.1% and 5.9% for method 2. For method 3 the recovery was 98.6% at the low concentration level, whereas recovery at the higher concentration level was 106%.

Table 1
Coefficients of variation for the determination of the concentration of 2,5-HD at two concentration levels

		Coefficient of variation in concentration (%)		
	n	Method 1	Method 2	Method 3
Concentration level 1[a]	10	5.8	5.3	2.7
Concentration level 2[b]	10	8.6	2.0	3.1

[a] Level 1: final concentration methods 1 and 2: 0.2 μmol/l, method 3: 2.0 μmol/l.
[b] Level 2: final concentration methods 1 and 2: 0.9 μmol/l, method 3: 8.6 μmol/l.

Matrix effect

For each individual urine sample, for all three methods, linear regression lines were constructed. The coefficients of variation for the slopes were 18.0%, 18.9% and 3.6% for method 1, 2 and 3, respectively. For method 3, the variation in slopes is in the same order of magnitude as the precision (Table 2). Both derivatization methods however, show a rather high discrepancy in slopes compared with the precision.

Bias from the added value

For each method, the determined value was plotted against the added value as shown in Figs. 2 and 3. In Fig. 2, samples spiked with a concentration below 1 μM are shown for methods 1 and 2. Samples spiked with a concentration in the range 1–10 μM are shown in Fig. 3. As

Table 2
Coefficient of variation in the slopes of the regression lines

	Method 1	Method 2	Method 3
Slope (average)	0.40	0.41	10.46
S.D.	0.072	0.078	0.38
C.V. (%)	18.0	18.9	3.6

Urine samples were spiked at two different concentration levels (final concentrations method 1: 0.30 and 0.59 μmol/l urine, method 2: 0.33 and 0.66 μmol/l urine, method 3: 3.31 and 6.62 μmol/l urine) in duplicate.

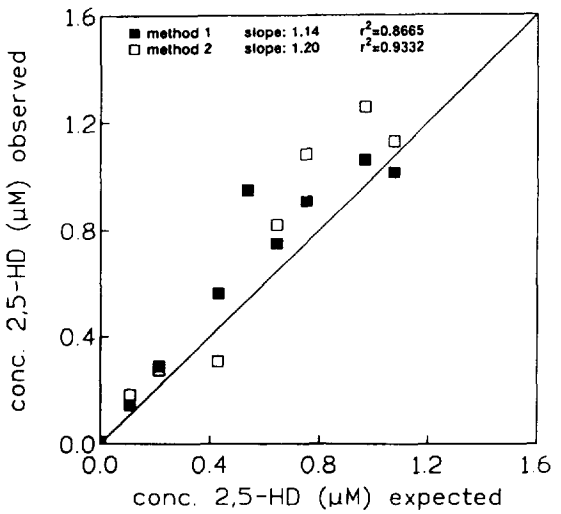

Fig 2. Concentrations of 2,5-hexanedione (2,5-HD) measured compared with concentrations expected as determined by methods 1 and 2. Concentration range, 0–1 μmol/l.

can be seen in Fig. 2, for both methods, the slopes of the regression lines are larger than one. As can be concluded from r^2, the deviation is relatively high. This deviation is not seen in Fig. 3, where samples from methods 1 and 2 with a

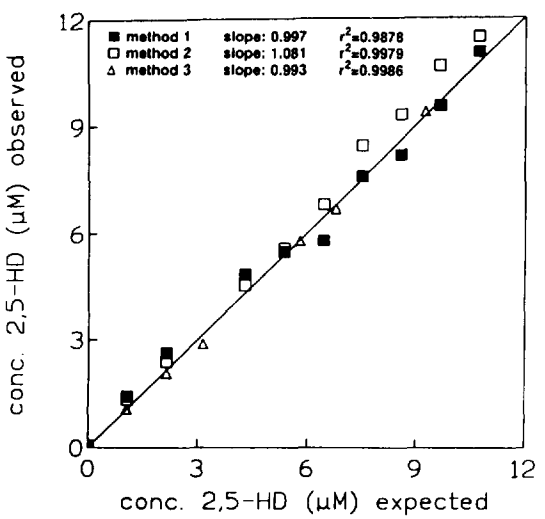

Fig 3. Concentrations of 2,5-hexanedione (2,5-HD) measured compared with concentrations expected as measured by methods 1, 2 and 3. Concentration range, 0–10 μmol/l. For methods 1 and 2 samples were diluted 10 times with pooled blank urine.

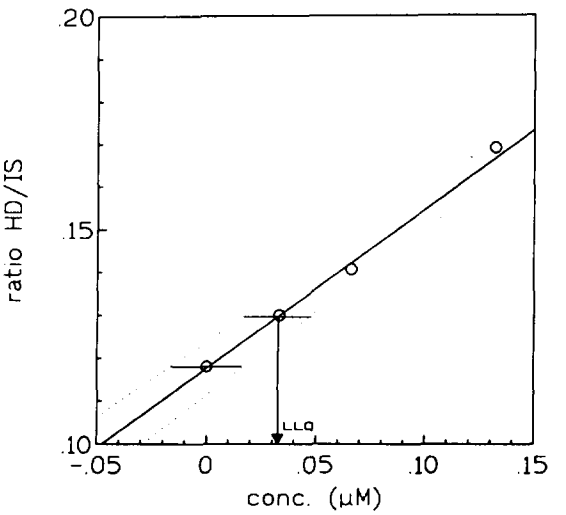

Fig 4. Estimation of the lower limit of quantitation (LLQ) for method 2. Dotted lines indicate the 95%-confidence interval for the ratio 2,5-HD/I.S. to spiked concentration. Horizontal bars indicate the 95%-confidence intervals for the blank value and the LLQ, respectively.

concentration above 1 μmol/l were diluted 10 times with pooled blank urine. The slopes are close to one.

3.3. Limit of quantitation

The lower limit of quantitation was calculated to be 55 nmol/l urine for method 1, 32 nmol/l for method 2 and 120 nmol/l for method 3. As demonstrated in Fig. 4, the lower limit of quantitation is the concentration at which the 95% confidence interval does not overlap with the confidence interval of the blank standard. It should be noted, however, that in the method 2 only 500 μl of urine was extracted, whereas in method 3 the urine sample volume was 5 ml.

4. Discussion

In the evaluation of the applicability of the methods for the determination of 2,5-HD in urine, a number of reliability parameters were assessed.

An important reliability parameter is preci-

sion, especially when small differences in concentrations have to be traced, e.g. in a kinetic study. For all three methods, precision was good, as the coefficient of variation of 10 determinations of the same sample was below 10%, both for the low and the high concentrations. As expected, method 3 showed the best precision, since this method is the most straightforward one; no variation is introduced by a derivatization step. Accuracy was assessed by recovery, bias from the true value and by the matrix effect. As is evident from Table 2, the coefficients of variation in the slopes of the regression lines for methods 1 and 2 are much higher than for method 3. For the latter, this variation is in the same order of magnitude as the variation determined at precision. The matrix effect in methods 1 and 2 is probably due to variations in the derivatization reaction depending on the composition of the individual matrix, rather than to variations in extraction or elution, as both recoveries were over 90%, and the C.V. for precision was less than 10%. However, no correlation with urinary pH or density could be demonstrated. The influence of the matrix could be avoided or minimized by diluting the matrix with e.g. pooled blank urine. This is also apparent from Figs. 2 and 3, in which the bias from the true (added) value is shown. As judged by the r^2 and the slopes of the regression lines, the deviation from the true value is considerably higher for samples with a concentration below 1 μM compared with concentrations exceeding 1 μM. Because of the limited linearity of the ECD, these latter samples were diluted 10 times with pooled blank urine, thereby minimizing the matrix effect. Method 3 showed a good correlation between the concentrations added and determined. The sensitivity of methods 1 and 2 was better than the sensitivity of method 3, owing to the introduction of F-atoms during derivatization and, therefore, the possibility to use the very sensitive electron-capture detection.

The sensitivity of method 2 could be easily improved by using a larger sample volume. High sensitivity is particularly important when low concentrations of 2,5-HD have to be determined or if only a small quantity of sample is available. In our study (Van Engelen et al., in preparation) where volunteers were exposed for 15 min to a low concentration of *n*-hexane [60 ppm (211 mg/m^3)], the concentration of 2,5-HD (in this case in blood) was lower than the limit of quantitation of method 3. The small quantity of the (blood) sample was another reason why method 3 was not suitable for application in the volunteer study. On the other hand, at occupational exposure, where 2,5-HD concentrations are much higher, the sensitivity of the rather simple method 3 is sufficient. This may not be true if the current threshold limit value (TLV; in the USA, for *n*-hexane the TLV is 176 mg/m^3) will be set at a lower level in the future, or if the concentration of 2,5-HD in blood is to be preferred for monitoring purposes.

In general, it can be concluded that all investigated methods meet the criteria for analytical reliability. It should be kept in mind, however, that a matrix effect might occur using methods 1 and 2, but this can be circumvented by diluting the urine samples with pooled blank urine. When the sensitivity or sample quantity is no point of concern, method 3 is the method of choice because of its simplicity, low costs and analytical reliability. The differences between method 1 and method 2 are of more practical relevance. The use of SPE may have certain advantages if a further concentration or cleaning step is necessary. The choice of eluent is less critical and automation of SPE is possible. On the other hand, method 2 is less expensive and less time-consuming, which is especially advantageous in case of a large number of samples.

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