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Microwave-assisted derivatization of 2,5-hexanedione in urine: evaluation using GC–MS and GC–ECD

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Abstract

2,5-Hexanedione, the main metabolite of *n*-hexane, can be responsible for axonal degeneration symptoms via formation of pyrrol-adducts with several amino acids. In order to make it amenable to gas chromatographic analysis, a protocol including microwave assisted derivatization is presented and compared to state-of-the-art technique of urine analysis. The applied methodology includes derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine, extraction of the oximes and final analysis using either GC–MS or GC–μECD. Furthermore, the mass spectra of derivatized 2,5-hexanedione and 5-hydroxy-2-hexanone as well as preliminary excretion kinetics are provided. Orthogonal regression methodology demonstrated superior sensitivity for the microwave heating. Limits of detection were calculated to be approximately 20 ng mL^{-1} with both MS and electron capture detection, the decompositon of excess derivatizing agent using sulfuric acid, following the reaction is beneficial. A matrix effect caused by urine was not observed, a calibration in aqueous matrix ensures accurate results therefore. Microwave heating yields excellent results regarding recovery, sensitivity and the time needed for sample preparation, furthermore, it is demonstrated that both mass selective as well as electron capture detection are of comparable suitability for this task.

Keywords: 2,5-Hexanedione; Microwave-assisted derivatization; Urine; GC–MS; GC–μECD

1. Introduction

Since the first observations of peripheral neuropathy caused by *n*-hexane and 2-hexanone at shoe workers in Japan, the United States of America and Italy in the 70's and 80's [1–4], extensive research has been conducted for the development of methods for the quantitative determination of these substances and their metabolites. One of the intermediates, 2,5-hexanedione (2,5-HD), was found to cause a neurotoxicity identical to that induced in experimental rats by inhalation of *n*-hexane [5]. Finally in 1980, 2,5-HD was proved to be the common intermediate of *n*-hexane and 2-hexanone [6]. The first step in the conversion of *n*-hexane to 2,5-HD takes place in either the lung with the end product mainly 2-hexanol and/or transport in intact state or as the phase 1 metabolite to the liver and further metabolization in a mixed-function oxidase system [7]. From a toxicological point of view, 2,5-HD is the most relevant metabolite and should therefore, be routinely determined in body fluids of workers occupationally exposed to such vapors. Its determination in urine is a fast and reliable way for gaining evidence of possible intoxication when conducted within 2–3 days following the uptake because of a relative fast renal excretion.

There is clear evidence of pyrrol-adduct formation with ε-lysin-residues of axonal proteins, oxidation and crosslinking of several filaments resulting in giant axonal swellings and degeneration [8,9]. Whether this axon accumulation is responsible for the neurotoxocity remains to be clarified [10,11].

Proposed biomarkers for past exposure to *n*-hexane are the already mentioned pyrrolidation of proteins or the evaluation of increased acetylcholine esterase release from tissues accompanying 2,5-HD intoxication [12,13].

Several methods are published for the determination of 2,5-HD in body fluids. It can be analyzed by gas chromatography, either directly after liquid–liquid extraction (LLE) with

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flame ionization detection [14] or after derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride followed by either solid phase extraction [15] or LLE [16] and quantitative analysis using mass spectrometry or electron capture detection. The work by Van Engelen et al. also shows a comparison of the last three methods [16]. The methods published until now require either for 12–16 h at room temperature [15,16] or up to 30 min at 65 °C [17]. Another approach uses 2,4,6-trichlorophenyl hydrazine at pH 1.5 followed by LLE [18] after acidic hydrolysis.

Most of the *n*-hexane metabolites, but not 2-hexanone and 2,5-HD, undergo phase 2 biotransformation to form glucuronides as a route of detoxification. Therefore, hydrolysis in acidic medium is used to cleave the conjugates for the quantitative determination of those metabolites. Already in 1987, Fedtke and Bolt [19] characterized 4,5-dihydroxy-2-hexanone as the main *n*-hexane metabolite in man, furthermore they specified the total conversion of that metabolite into 2,5-HD during the routinely applied acidic hydrolysis conditions. Those findings necessitate a differentiation between “free” and “total” 2,5-HD.

At least two reasons are of interest for the determination of both parameters: since 2,5-HD is the most toxic metabolite of *n*-hexane due to its capability to form pyrrol-adducts, the concentration of free 2,5-HD can be used as a biomarker of effect if the correlation between extent of neuronal crosslinking and toxicity can be verified. Total 2,5-HD can serve as a biomarker of exposure since a good correlation between the *n*-hexane concentrations in air during exposure, its concentration in blood and the amount of 2,5-HD in urine following acidic hydrolysis has been described [20]. Another metabolite of *n*-hexane being a direct precursor of 2,5-HD is 5-hydroxy-2-hexanone [21], a substance not detectable by GC due to dehydration and cyclization [22]. It was identified in hydrolyzed rat urine using APCI–LC–MS by Manini et al. [23].

Since the “free 2,5-HD” was considered to be the more important parameter, the determination in urine samples was performed without acidic hydrolysis. After exposure to hexane by sniffing for 3 min, the excretion was observed by means of derivatization, liquid–liquid extraction with cyclohexane and quantitative analysis applying combined gas chromatography–mass spectrometry or electron capture detection.

The objective of the present study was the application of a microwave accelerated derivatization reaction with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine to minimize the time needed for sample preparation. A further advantage of microwave heating is usually a reduced susceptibility for the formation of artefacts due to shorter reaction times. Microwave irradiation has already been applied in our laboratory for the derivatization of aldehyes and ketones in different matrices and proved to be superior to conventional reactions in terms of effectiveness, recovery and preservation of organic compounds [24]. Consequently, also for the derivatization of 2,5-HD the usefulness of microwave heating was compared to that of conventional reaction systems. In a preliminary experiment, two published experimental settings (20 h at room temperature and 30 min at 65 °C) were evaluated with respect to their effectiveness for this purpose. Possible matrix effects due to the composition of urine were checked by comparing the calibration data in both spiked urine and water samples. Preliminary excretion kinetics are established by tracing urinary 2,5-HD concentrations over a time course of 7 h. To provide also the electron impact mass spectrum of derivatized 5-hydroxy-2-hexanone, the urine samples were hydrolyzed under acidic conditions.

2. Materials and methods

2.1. Chemicals

n-Hexane ultra resi-analyzed for inhalation purposes was purchased from J.T. Baker (Mallinckrodt Baker B.V., Deventer, Holland). 2,5-Hexanedione as well as 2,5-pentanedione were of quality for synthesis and were obtained from Merck-Schuchardt (Hohenbrunn, Germany), while *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA.HCl) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria). CH_2Cl_2, cyclohexane, H_2SO_4 37%, Na_2SO_4, K_2CO_3, HCl 37%, tri-sodium citrate 2-hydrate and citric acid monohydrate were of quality p.a. and obtained from Merck KgaA (Darmstadt, Germany).

2.2. Instrumentation and conditions of analysis

Volumes up to 100 μL were handled by means of calibrated capillaries, for larger volumes up to 5 mL Transferpettes from Brandt (Wertheim/Main, Germany) were used. The microwave reactions were carried out in 11 mL vials with PTFE-lined screw caps (Pyrex, UK) which where placed in water filled TFM/ceramics protective vessels (Anton Paar GmbH, Graz, Austria) for safety reasons. Six samples each were placed in the multiwave microwave sample preparation system (Anton Paar GmbH, Graz, Austria).

All GC analyses were accomplished with Agilent Model 6890 GCs equipped with Agilent 7683 series injectors. The pentafluorobenzyloximes were detected by mass spectrometry with an Agilent 5973 mass selective detector with electron impact ionization as well as with an Agilent G2397A μ-electron capture detector.

The column was an Agilent HP5-MS 30 m × 0.25 mm × 0.25 μm fused silica capillary. Helium 5.0 (Air Liquide, Graz, Austria) was used as carrier gas at constant flow of 1.1 mL min^{-1} with a pressure pulse of 150 kPa for 0.5 min at injection. The gas chromatograph was programmed to keep an initial temperature of 110 °C for 1 min and to ramp at a rate of 12 °C min^{-1} to a temperature of 250 °C and to further increase the temperature at a rate of 25 °C to a final temperature of 300 °C which was held for 3 min. Injection was set to 1 μL in splitless mode.

Regarding mass spectrometric detection single ion monitoring mode was used for the quantitation of bis-PFB-2,5-hexanedione recording *m*/*z* values of 96, 181, 292, and 307, while *m*/*z* 181, 236 and 293 were recorded for the internal standard 2,4-pentanedione.

Data acquisition was carried out with an Agilent Chemstation G1701BA.

For further evaluation and preliminary determinations Microsoft-Excel (V 7.0), Statgraphics Plus 3.0 (Manugistics, Rockville, MD, USA) and the Excel-macro ValiData v3.02.54 (©Wegscheider, Rohrer, Neuböck, Leoben, Austria), which permits to calculate analytical performance data according to the Eurochem/CITAC Guide 2000, were used.

2.3. Samples and standards

Calibration was accomplished in a concentration range between 20 and 500 ng mL^{-1} using three steps. For the evaluation of possible matrix effects from urine, both water and pooled blank urine were spiked with four different concentrations between 24 and 600 ng mL^{-1}. 2,4-Pentanedione (2,4-PD) was used as internal standard to yield a final concentration of 254 ng mL^{-1}. The reaction media consisted of 5 mL sample volume, with an adjustment to pH 5 using 0.5 mL of citrate-puffer (21 g citric acid monohydrate and 29 g tri-sodium citrate 2-hydrate in 100 mL H_2O bidest., adjusting a pH of 3.92 ± 0.03 at 25 °C), while 2 mg of PFBOA in 1 mL of bidistilled water were added as derivatization agent. The conditions used in the preliminary examinations for the conventional reaction were 20 h reaction time at room temperature and 30 min at 65 °C, respectively. The microwave enhanced reactions were carried out in the multiwave sample preparation system, which was operated at 600 W for 5 min with the maximum internal temperature set to 110 °C followed by active cooling for 15 min. In any case, the excess of derivatization agent was decomposed by adding one drop of H_2SO_4 37%, afterwards the oximes were extracted twice with 1 mL of cyclohexane, the merged organic phases were combined in a 4 mL glass vial and dried by addition of 250 mg anhydrous Na_2SO_4. After vigorously shaking the clear organic phase was directly injected into the GC–MS.

For establishing the excretion kinetics and the structural elucidation of the metabolites, the volunteer was exposed to *n*-hexane vapors for 3 min until a slight vertigo could be observed. During a time course of 7 h, seven samples were collected with one just before exposure in order to determine the blank urine value. After addition of the internal standard 2,4-PD to 5 mL of urine to give a final concentration of 254 ng mL^{-1}, derivatization was conducted applying microwave irradiation operating at the conditions mentioned above.

Mild acid hydrolysis was applied for the identification of 5-hydroxy-2-hexanone in human urine samples. For this purpose 2 mL urine were mixed with 100 μL of HCl 37% and were kept in a boiling water bath for 10 min. After neutralization of the reaction mixture with K_2CO_3 (10% in H_2O bidest.), the analytes were extracted twice with 1 mL CH_2Cl_2 each which was then evaporated with a gentle stream of nitrogen. The residue was redissolved in 4 mL of methanol. Following derivatization and further sample processing, the oximes were extracted with CH_2Cl_2, which was found to result in highest recovery of bifunctional carbonyls (as also reported by Spaulding and Charles [25]). After evaporation to dryness, 100 μL of MSTFA were added. Trimethylsilylation was accomplished during 30 min at 60 °C. A N_2-flow was used to remove the excess solvent, then 1 mL of *n*-hexane was added to redissolve the derivatives.

Standard solutions for calibration purposes were prepared in a concentration range from 24 to 600 ng mL^{-1} in H_2O dest. and pooled blank urine respectively and treated the same way as the samples.

3. Results and discussion

3.1. Preliminary experiments

In order to establish proper conditions for derivatization by conventional heat transfer, two key parameter assays using either 20 h of reaction time at room temperature or 30 min at 65 °C on a hot plate were evaluated. Both procedures were compared using a robust regression procedure, which indicated about 30% higher recovery at elevated temperatures. Therefore, these conditions were used as a reference for evaluating the potential of microwave heating.

3.2. Comparison conventional/microwave heating

Two data arrays resulting from the derivatization of 20–500 ng mL^{-1} of 2,5-HD using both 65 °C for 30 min and microwave heating for 5 min were compared applying a robust regression methodology. For demonstration purposes, the two calibration graphs are displayed in Fig. 1. Since the slope of the graph corresponding to microwave heating excels that resulting from conventional heating, the superiority of the new derivatization process regarding both recovery and sensitivity is in clear evidence. Unfortunately, a calculation of the derivatization efficiency was not possible, due to the characteristic of these analytical problem. The

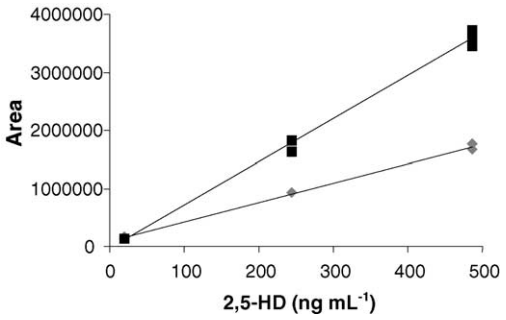

Fig. 1. Calibration graphs resulting from derivatization using conventional (below) and microwave (above) heating.

underivatized 2,5-HD as well as 2,4-PD are not directly amenable to GC-measurement, therefore, the disappearance of the starting material could not be determined. Owing to a leak in commercially available pure derivatives of both compounds of interest, an authentic, external calibration could not be carried out. Nevertheless, the reaction efficiency of the derivatization process should be high to yield low limits of detection. Therefore, the microwave-assisted derivatization procedure with respect to the peak area of the 2,5-HD derivative had been optimized by a surface response design investigating microwave energy, reaction time and temperature [26]. Using the same analytical protocol including the derivatization procedure for samples and standard solutions—both containing also the internal standard—a reliable quantification is guaranteed.

As already mentioned, the excess of PFBOA was decomposed prior the liquid–liquid extraction by adding one drop of concentrated sulfuric acid. In order to verify the efficiency of this work step, the whole methodology was conducted on two parallel samples, with and without addition of the sulfuric acid. The effect of sulfuric acid onto decomposition of the reagent is visualized by means of comparison of two GC–ECD chromatograms in Fig. 2. Decomposition of excess PFBOA results in obviously cleaner extracts, thus providing also for a robust environment for the chromatographic separation system.

3.3. Calibration in urine and water matrix–matrix effects

Furthermore, a possible matrix effect of urine was checked by applying the whole methodology to both spiked water and urine samples at four concentration levels (24–600 ng mL^{-1}). Orthogonal regression was used to compare the resulting calibration data as shown in Fig. 3. The slope of the calibration graph approximating unity (0.97) indicates independence of the applied calibration methodology from the composition of the matrix within the limits of the parameters. Therefore, it is reliable to prepare the calibration solutions for quantification in water matrix.

3.4. Evaluation of detector performance

The conversion of carbonyls into their corresponding pentafluorobenzyloximes favours the application of several selective gas chromatographic detectors. Self-evident is the

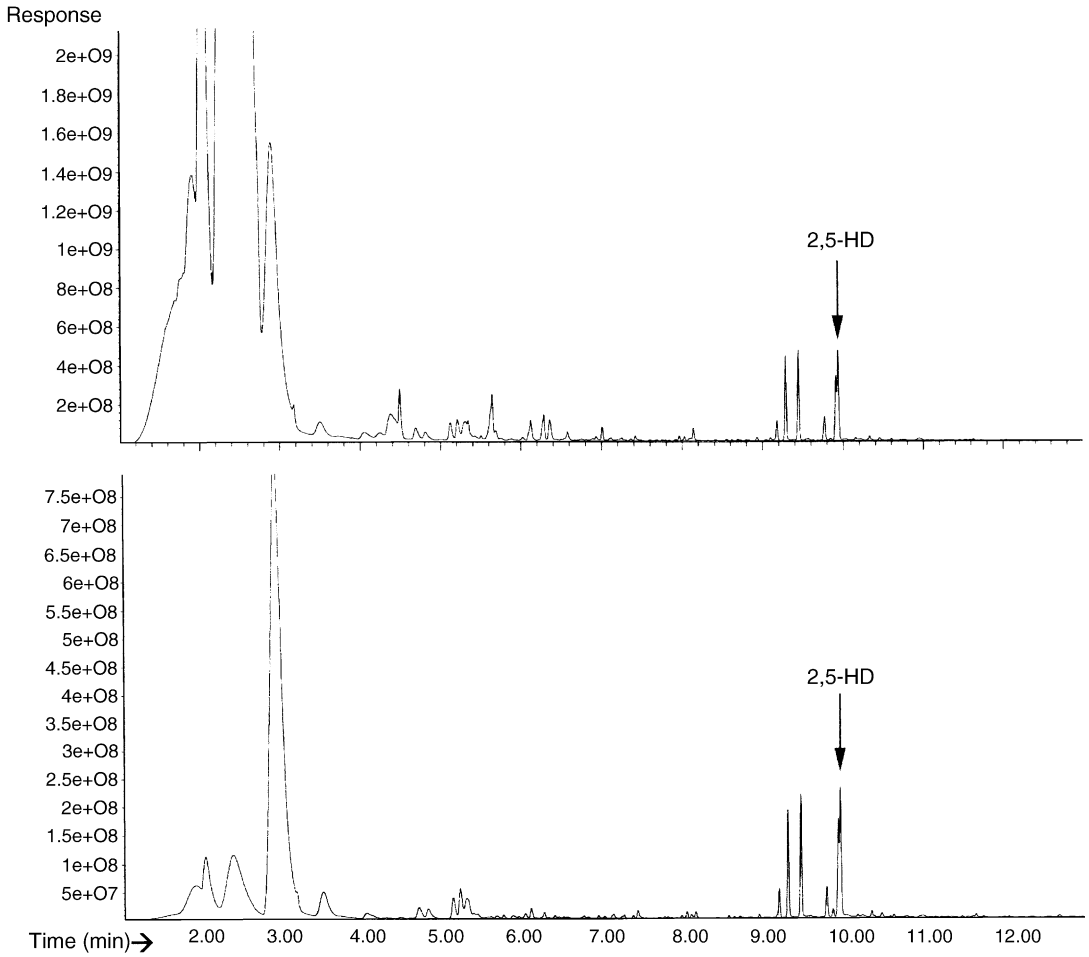

Fig. 2. Effect of excess PFBOA-reagent using sulfuric acid. Displayed are GC–ECD chromatograms without (above) and with (below) the decomposition step, the labels indicate the signals of 2,5-HD (100 ng/mL).

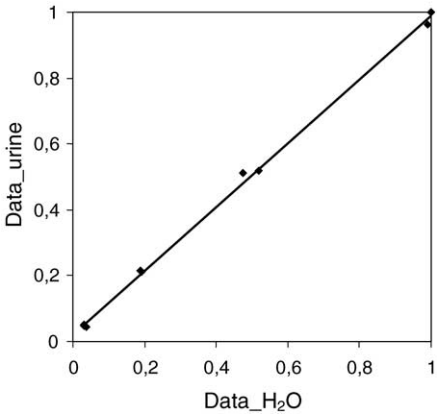

Fig. 3. Orthogonal regression graph obtained for evaluating a possible effect caused by urine matrix onto the derivatization step using PFBOA. Calibration in water is referred to be the reference method (Data_H_2O), while the ordinate specifies the results in urine matrix using internal standard calibration.

possibility to use electron capture detection due to five fluorine-atoms per carbonyl-residue, as well as the potential of mass spectroscopy, especially in single ion monitoring mode. In order to provide a means of comparison between these two detectors, calibrations were performed using both MSD and μECD. Fig. 4 shows the reconstructed total ion chromatogram of an urine sample. The double peaks caused by *E*/*Z*-configuration at the oxime bond of both the internal standard as well as analyte are visible. For quantification, the peak areas of the *E*/*Z*-conformers were summed. The chromatogram obtained with electron capture detection is depicted in Fig. 5. For comparison purposes, the appropriate time slot is enlarged and reveals the resemblance of the two detectors. Also, the calculated limits of detection are comparable in the range of approximately 20 ng mL^{-1}.

Furthermore, other characteristic analytical performance data of the method were determined. The limit of detection was calculated from the calibration data from the matrix comparison experiments by means of the Excel-Macro Validata at the 95% confidence interval and equals 17 ng mL^{-1} for mass selective detection and 22 ng mL^{-1} for electron capture detection, while the repeatability was determined from 10 parallel samples at a concentration level of 121 ng mL^{-1} and was calculated to be 4% with both detectors.

3.5. *n*-Hexane metabolites from urine—GC–MS

The metabolites of *n*-hexane in urine were qualitatively identified applying mass spectrometry in scan mode with *m*/*z* from 70 to 500. 5-Hydroxy-2-hexanone, reportedly to be not detectable in its free form by gas chromatography [22], was found in remarkable amounts after acid hydrolysis. Typical chromatograms with and without acidic hydrolysis have been published already and can be found in literature [27]. In Fig. 6, the mass spectrum and structure of 5-hydroxy-2-hexanone and the proposed sites of cleavage during electron impact ionization are shown. A mass charge ratio *m*/*z* 73 corresponds to –($Si(CH_3)_3$), a fragment always observable

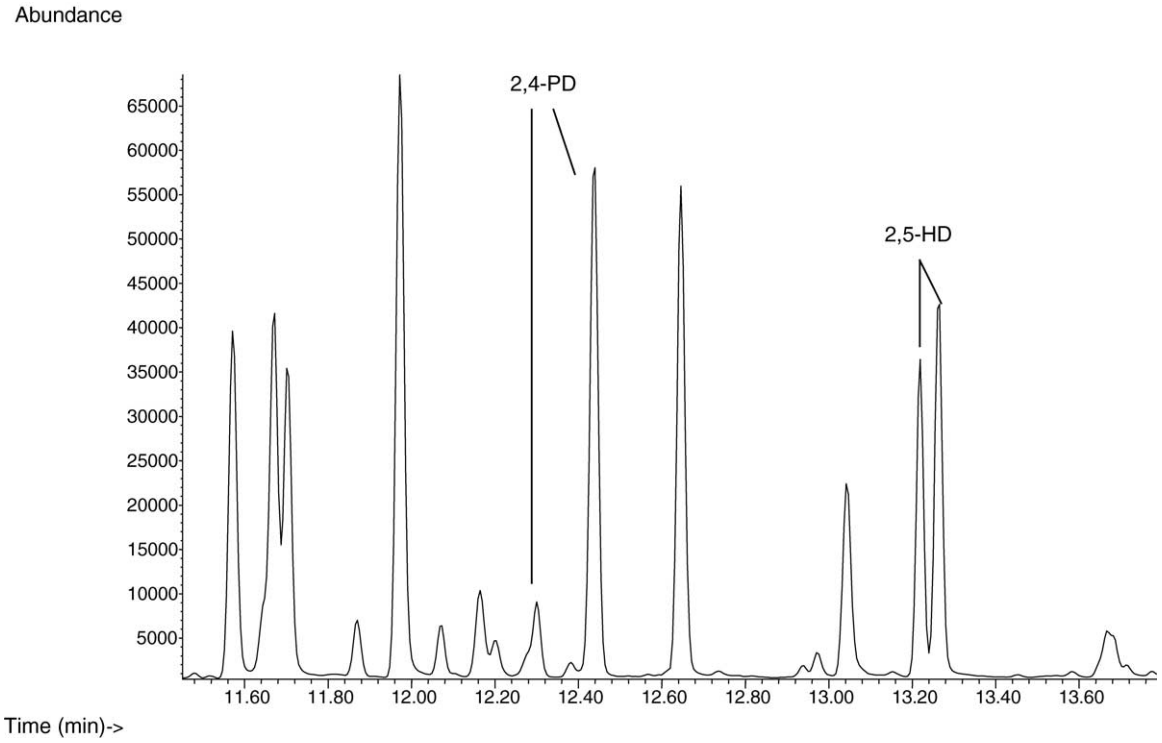

Fig. 4. Reconstructed mass spectrometric total ion chromatogram (TIC) of a derivatized urine sample. Indicated are the internal standard as well as 2,5-HD, both signals correspond to 122 ng/mL.

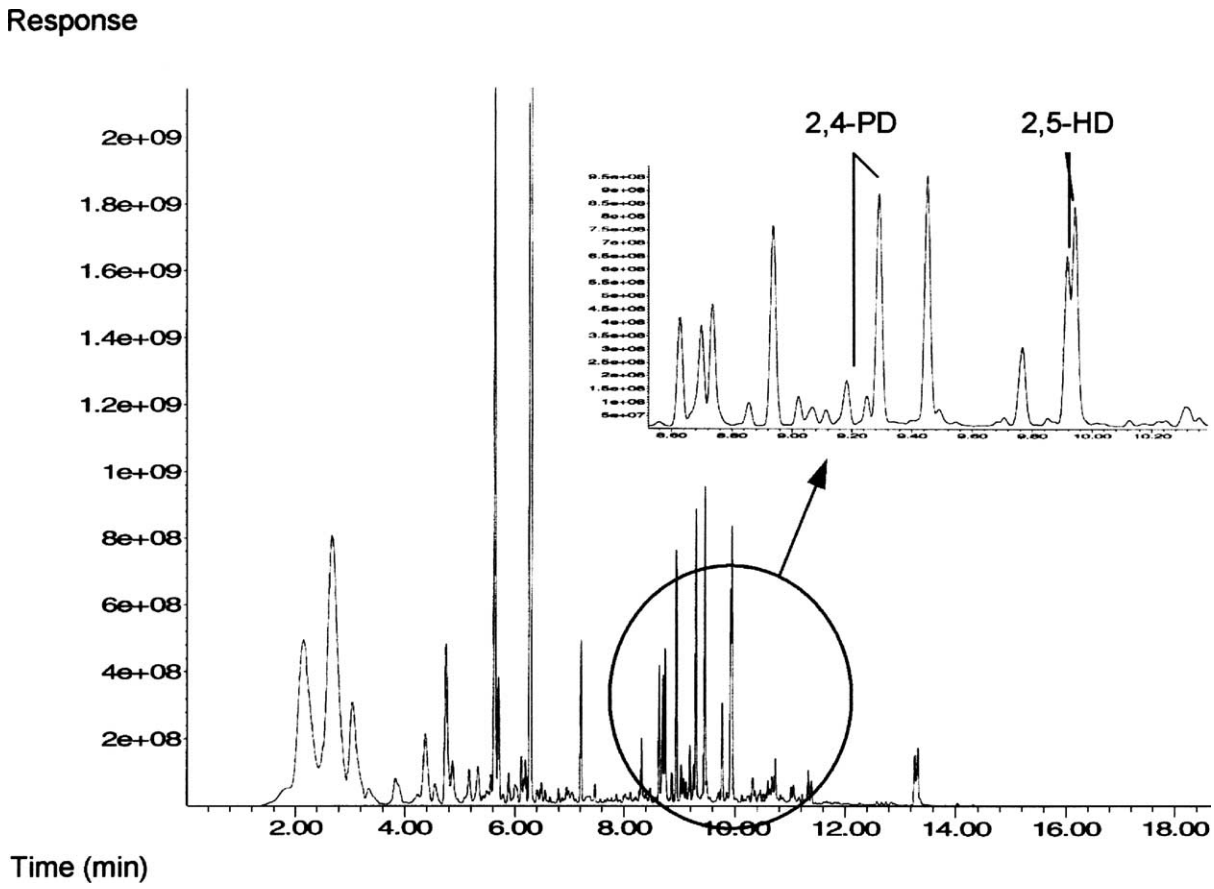

Fig. 5. GC–μECD chromatogram of the same sample as shown in Fig. 4. The interesting area of the chromatogram is shown in the small illustration, the similarity to the TIC obtained with the MSD is obvious.

in trimethylsilylated compounds. Furthermore, visible is the loss of a methyl residue with *m*/*z* 15 resulting in *m*/*z* 368, while *m*/*z* 181 corresponds to the pentafluorobenzyl-ion –(CH_2–C_6F_5). Also the molecular ion with *m*/*z* 383 can be clearly detected. The electron impact mass spectrum of the bis-pentafluorobenzyloxime of 2,5-HD exhibits a fragmentation pattern as shown in Fig. 7. Again, the molecular ion with *m*/*z* 504 is visible, the sequence *m*/*z* 323, 307 (not indicated) and 292 corresponds to the successive loss of –(CH_2–C_6F_5), –(O–CH_2–C_6F_5) and –(N–O–CH_2–C_6F_5). Additional repulsion of –(O–CH_2–C_6F_5) from the fragment with *m*/*z* 292 could result in *m*/*z* 96.

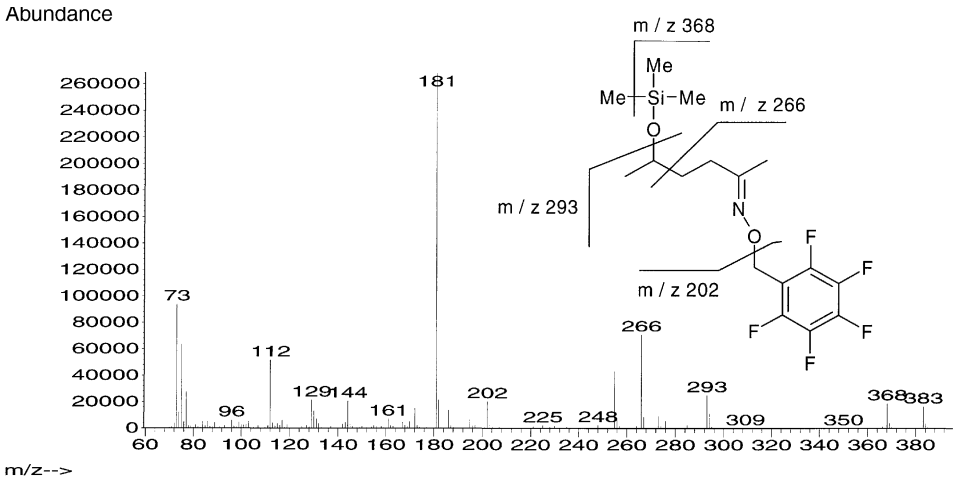

Fig. 6. Electron impact mass spectrum and proposed origin of fragments of the 5-trimethylsilyloxy-2-hexanone as the pentafluorobenzyloxime derivative (MW 383).

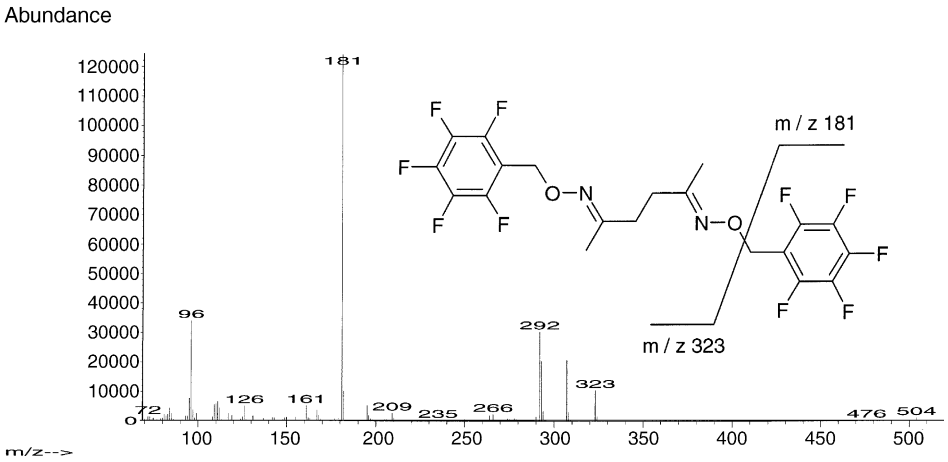

Fig. 7. Electron impact mass spectrum, structure and proposed fragmentation sites of the bis-PFB-derivative of 2,5-hexanedione (MW 504).

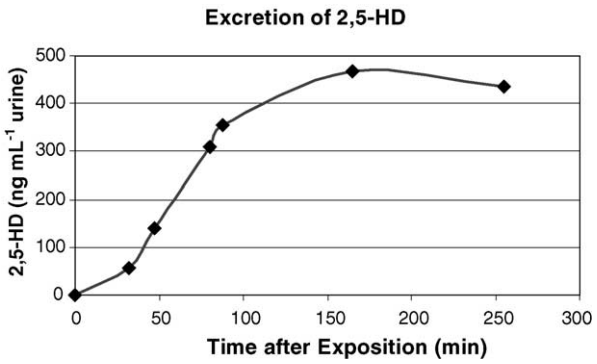

Fig. 8. 2,5-Hexanedione (2,5-HD) concentration in urine versus time after exposition.

3.6. n-Hexane exposure: metabolite concentration in urine

In order to establish an excretion kinetics for the leading metabolite 2,5-HD a volunteer was exposed to *n*-hexane vapors. During a time course of about 7 h seven samples were collected with one just before exposure. This periodical sampling was applied for a very first estimation of the kinetics for the *n*-hexane metabolism. For a more detailed approach standardization of the excretion rates from urine, e.g., by correction on the creatinine-concentration or by density measurements, is mandatory. The measured excretion kinetics for the leading substance 2,5-HD is given in Fig. 8. As can be seen from the graph, after an instantaneous increase a maximum is reached after about 3 h.

4. Conclusion

An improved method for the determination of 2,5-hexanedione in urine applying a microwave-accelerated derivatization reaction using *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride has been developed. Since 2,5-HD does not undergo phase 2 biotransformation and is not excreted as a glucoronide conjugate therefore, the acidic hydrolysis step proposed in literature was omitted to guarantee accurate results. After derivatization in buffered medium using 2,4-pentanedione as an internal standard, the resulting oximes are extracted twice with cyclohexane and determined using either GC–MS or GC–μECD. Both detectors proved to be of comparable suitability for this particular task. The performance of the optimized microwave enhanced method was compared with conventional heating and proved to be superior with respect to reaction time and recovery. The need for a decomposition of excess derivatization agent with sulfuric acid became evident. Robust regression was used to prove the independence of the analytical results of the matrix composition within regular limits. Additionally, acid hydrolysis was used to provide the fragmentation pattern of 5-hydroxy-2-hexanone, the proposed direct precursor of 2,5-HD, as its pentafluorobenzyl-trimethylsilyl-derivative.

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