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Chromatographic determination of amines in biological fluids with special reference to the biological monitoring of isocyanates and amines

IV. Determination of 1,6-hexamethylenediamine in human urine using capillary gas chromatography and selective ion monitoring

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ABSTRACT

A capillary gas chromatographic (GC) method was developed for the determination of 1,6-hexamethylenediamine (HDA) in hydrolysed human urine. The method was based on a derivatization procedure with heptafluorobutyric anhydride. The amides formed were determined using capillary GC with selected ion monitoring in the chemical ionization mode with ammonia as reagent gas. The overall recovery was 34% for a concentration of 100 μg/l of HDA in urine. The minimum detectable concentration in urine was below 0.5 μg/l. The precision of the method was 5% (n = 9). Deuterium-labelled HDA [$H_2NC^2H_2(CH_2)_4C^2H_2NH_2$] was used as the internal standard. A male subject was exposed to hexamethylene diisocyanate (HDI) for 7.5 h in a test chamber. The average air concentration of HDI was *ca.* 30 μg/m^3, which corresponds to *ca.* 85% of the threshold limit value in Sweden (35 μg/m^3). The half time of urinary levels of HDA was *ca.* 1.4 h and more than 90% of the urinary elimination was completed within 4 h after the exposure. The amount of HDA excreted in urine was *ca.* 10 μg, corresponding to *ca.* 10% of the estimated inhaled dose of HDI.

INTRODUCTION

1,6-Hexamethylenediamine (HDA) is an aliphatic diamine of industrial importance. HDA is used as raw material for nylon 66, and in the manufacture of urethane coatings and polyamides. The compound is also used in paints and as a curing agent for epoxy resins[1]. HDA is moderately toxic[2–4], but has been associated with health

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hazards in the work environment[5]. In addition, HDA has been determined in air after pyrolysis of lacquered metal parts with a lacquer-resin based on 1,6-hexamethylene diisocyanate (HDI)[6].

Measurements of the concentrations of chemicals in the air in the breathing zone give only fragmentary information about the absorbed dose, and do not ensure that the worker is totally protected from adverse health effects. Biological monitoring integrates exposure by all routes (*e.g.*, pulmonary, oral and skin) and from all sources. It also consider individual factors such, as work load habits and genetic differences in biotransformation.

The possibility of using urine analyses for diamines as a test for occupational diisocyanate exposure was proposed by Rosenberg and Savolainen[7] and Brorson *et al.*[8].

Methods for the determination of 2,4- and 2,6-diaminotoluene and 4,4'-methylenedianiline (MDA) in human urine using gas chromatography–mass spectrometry (GC–MS) were described in Parts II and III in this series[9,10]. Deuterium-labelled internal standards were used. The biological monitoring of inhalation exposure to 2,4- and 2,6-toluenediisocyanate and skin exposure to MDA, respectively, were the purposes of those studies.

Methods for the determination of HDA, at mg/l levels in plasma, have been described by Egorin *et al.*[11]. Recently, a method based on a two-phase derivatization procedure with ethyl chloroformate was developed in our laboratory[12]. Capillary GC with thermionic specific detection (GC–TSD) made it possible to determine low concentrations (10–1000 μg/l) of HDA in urine after oral administration of the compound.

However, the potential use of HDA as a marker of HDI exposure made it necessary to develop an even more sensitive method. The aim of this study was to develop a method for the determination of HDA in trace amounts (μg/l) in complex matrixes such as biological fluids.

EXPERIMENTAL

Apparatus

A Shimadzu (Kyoto, Japan) GCMS-QP 1000 EI/CI quadrupole mass spectrometer connected to a Shimadzu GC-9A gas chromatograph equipped with a split/splitless injection system SPL-G9 and a Shimadzu autosampler (AOC-9) was used.

The starting temperature of the column oven was set near to the boiling point of the solvent, and for toluene it was 100°C isothermal for 2 min. The split exit valve was kept closed for 1 min after injection. After elution of the solvent, the column was programmed at 30°C/min to 280°C, where it was maintained for 1 min. The capillary column outlet was mounted directly in the ion source. The gas chromatograph–mass spectrometer interface and the ion source were held at 250°C. Chemical ionization was utilized with ammonia and isobutane as reagent gases. Samples were introduced into the chromatographic system with an autosampler using a splitless technique at 250°C. The carrier gas was helium and the inlet pressure was 1 kg/cm^2. The amount injected was typically 4 μl, using a Hamilton 701RN syringe with a point style 5 needle with a conical point and a side-hole to minimize septum coring.

For enrichment and evaporation of solvent a vacuum desiccator connected to an aspirating pump was used. The apparatus was equipped with an electrically heated oven, designed and manufactured at our laboratory. A Sigma 3E-1 centrifuge (Sigma, Harz, F.R.G.) was employed for phase separation.

Columns

A fused-silica capillary column (30 m × 0.247 mm I.D.) coated with DB-5 bonded stationary phase (J & W Scientific, Folsom, CA, U.S.A.) with a film thickness of 0.25 μm was used.

Chemicals

Chemicals used were 1,6-hexamethylenediamine (HDA) and toluene from Janssen (Beerse, Belgium), heptafluorobutyric anhydride (HFBA) from Pierce (Rockford, IL, U.S.A.), deuterium-labelled HDA [$H_2NC^2H_2(CH_2)_4C^2H_2NH_2$] from MSD Isotopes (Merck Frosst Canada, Montreal, Canada), HCl, NaOH and K_2HPO_4 from Merck (Darmstadt, F.R.G.)

Synthesis of 1,6-hexamethylenediheptafluorobutyramide

After recrystallization of HDA from toluene, 0.2 g (1.6 mmol) of the amine and 1.6 g (4 mmol) of HFBA were dissolved in ethyl acetate and the solution was heated at 50°C for 10 min. After cooling to room temperature, the excess of reagent and liberated acids were extracted with a 1 *M* phosphate buffer solution (pH 7.0) and the organic phase was eluted through a silica column with ethyl acetate. Evaporation of the ethyl acetate to dryness gave a *ca.* 500 mg (80%) yield of the amide (HDA–HFBA).

Preparation of standard solutions

Standard solutions of the HDA–HFBA derivatives was prepared by dissolving accurately weighed amounts in toluene. The solutions were then further diluted with toluene to the appropriate concentrations. Standard solutions of HDA and deuterium-labelled HDA were prepared and stored as 6 *M* HCl solutions.

Sampling and storage of urine samples

Urine samples were acidified by the addition of 5 ml of 6 *M* HCl per *ca.* 100 ml of urine. The urine samples were stored in a refrigerator at 4°C until analysis.

Work-up procedure

A 1.5-ml volume of 6 *M* HCl and 1.5 ml of 6 *M* HCl containing the internal standard (*ca.* 6 μg/l), were added to a 2-ml urine sample. The mixture was heated at 100°C overnight for hydrolysis. After cooling to room temperature, a 2-ml aliquot was transferred to a test-tube and 4 ml of saturated NaOH and 3 ml of toluene was added. HDA was extracted into the organic phase by shaking the mixture for 5 min. The sample was then centrifuged at 1500 *g* for 10 min. A 2-ml volume of the organic layer was transferred into a 10-ml test-tube and 20 μl of HFBA were added. The mixture was immediately shaken vigorously for 5 min on a Vortex mixer. The excess of reagent and the acid formed were extracted for 10 min with 2 ml of 1 *M* phosphate buffer solution (pH 7.5). The toluene layer, containing the amide formed, was then transferred into a test-tube. The toluene phase was evaporated in a vacuum desiccator at 30°C and the

dry residue was dissolved in 50 μl of toluene. A volume of 4 μl was then injected into the GC–MS system. Duplicate analyses and injections were made.

RESULTS AND DISCUSSION

Standard

The identity of the HDA–HFBA derivative was confirmed by GC–MS, and the purity was determined using capillary GC–TSD and GC with flame ionization detection. The purity was further examined by elemental analysis, and was found to be higher than 99%.

Internal standard

The determinations by GC–MS were performed using tetradeuterated HDA as the internal standard. The hydrogen atoms in the α-positions to the amine groups were exchanged with deuterium atoms. Several advantages of using the deuterium-labelled compound as internal standard were found. The chromatographic and chemical properties were found to be similar to those of HDA, which was very important owing to the low overall recovery. No interferences were found for the mass fragments monitored using chemical ionization and the similar ionization patterns of HDA and tetradeuterated HDA were favourable for easy calibration of the mass spectrometer. Finally, tetradeuterated HDA was not expected to occur as an interferent in the sample.

Work-up procedure

Storage and treatment of samples. HDA-spiked urine samples were found to be stable after acidification. No noticeable change in the sample composition was observed when stored in darkness at room temperature for several weeks.

Hydrolysis. No losses were found in the hydrolysis step for HDA-spiked urine. The sum of free HDA and hydrolysable HDA conjugates was determined.

Derivatization. A two-phase derivatization of aliphatic diamines with HFBA has earlier been developed for aqueous solutions. For urine samples the recoveries were very low, and not reproducible[13]. In this study, the derivatization procedure was therefore performed in toluene, after extraction of aqueous urine hydrolysates. The excess of reagent and liberated acid were removed by extraction with a 1 *M* phosphate buffer solution (pH 7.5), without any measurable loss of the HDA amide derivative.

Enrichment. A 1-ml volume of the organic phase containing the amide derivative was evaporated to dryness and the residue dissolved in 50 μl of toluene, giving a 50-fold enrichment. The recovery for the evaporation and enrichment step was *ca.* 100%.

Choice of reagent

Derivatization reagents such as trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA), acetic anhydride (AA), ethyl chloroformate and isobutyl chloroformate were tested, giving *ca.* 100% recovery. All the reagents tested could be used for a single-phase derivatization. With the exception of the AA derivatives, all the tested derivatives were found to have satisfactory chromatographic behaviour. However, the sensitivity of the GC–selected ion monitoring (SIM) system was much better for the perfluoro fatty acid

derivatives. The HDA–HFBA derivative showed a slightly better sensitivity and better resolution relative the matrix, and the HDA–HFBA derivative was therefore chosen.

Mass spectrometry

Electron impact (EI) with ionization potential 70 eV and chemical ionization (CI) with ammonia or isobutane were investigated (Fig. 1). It was established that several components in the sample were derivatized with the acylating reagent. Selective detection was therefore necessary. The sensitivity in the EI mode was relatively low, and *ca.* 50 ng were needed to give an acceptable spectrum when injecting 1 μl of standard solution. The relative abundance of the molecular ion was only *ca.* 1%. Owing to the relative abundance, the fragment ions of *m/z* 339 and 343 ($M^+ - C_3F_7$) were chosen for the SIM of HDA–HFBA and tetradeuterated HDA–HFBA. When analysing spiked urine samples, baseline separation of the investigated compounds relative to the matrix could not be achieved.

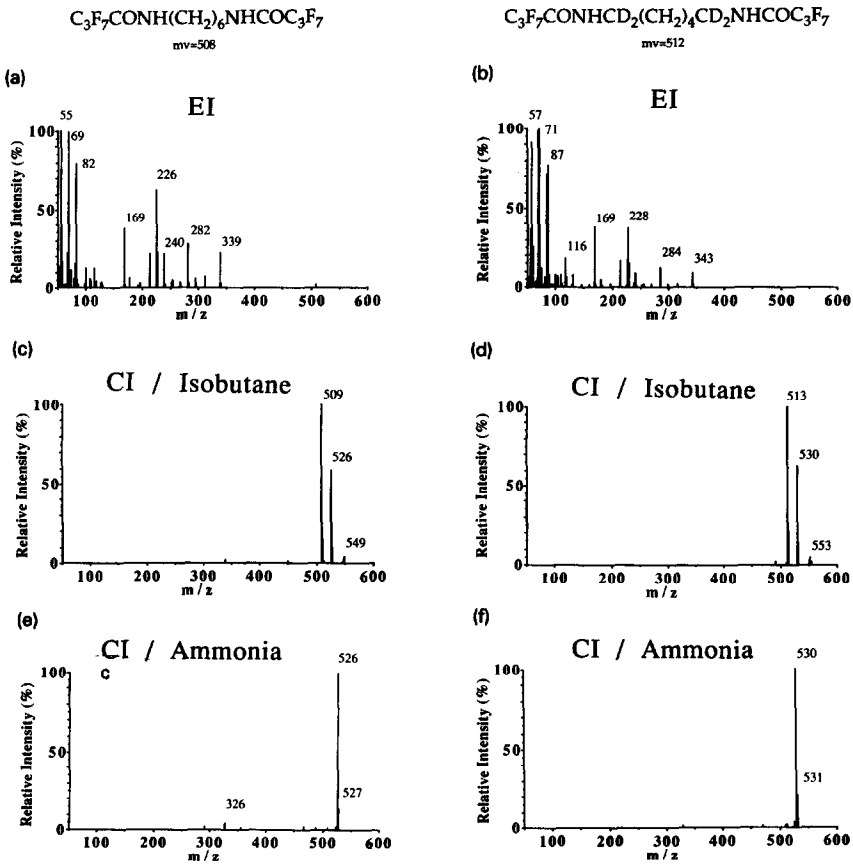

Fig. 1. Mass spectra obtained with electron impact (70 eV) for (a) HDA–HFBA derivative and (b) deuterium-labelled HDA–HFBA derivative, chemical ionization using isobutane for (c) HDA–HFBA derivative and (d) deuterium-labelled HDA–HFBA derivative and chemical ionization using ammonia for (e) HDA–HFBA derivative and (f) deuterium-labelled HDA–HFBA derivative. The amount injected was *ca.* 70 ng. mv = Molecular weight.

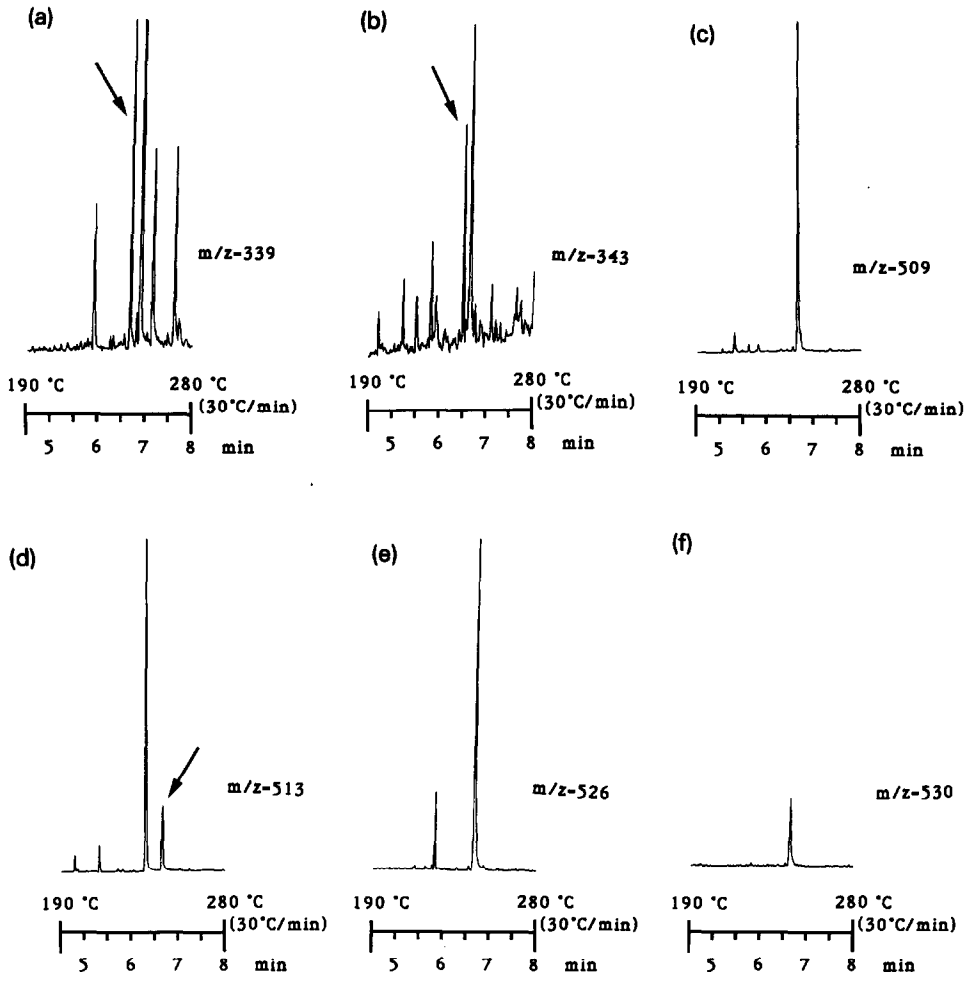

Fig. 2. Selected ion monitoring of HDA in hydrolysed urine samples using electron impact and chemical ionization. Chromatogram (a) shows EI and m/z = 339, (c) shows CI using isobutane and m/z = 509 (M + 1) and (e) shows CI using ammonia and m/z = 526 (M + 18) for a urine sample from a subject exposed for 7.5 h to HDI (the HDI air concentration was *ca.* 30 $\mu g/m^3$). The peaks in the chromatograms correspond to a concentration of *ca.* 20 μg of HDA per litre of hydrolysed urine. Chromatogram (b) shows EI and SIM, m/z = 343, (d) shows CI using isobutane, m/z = 513 (M + 1), and (f) shows CI using ammonia, m/z = 530 (M + 18), for deuterium-labelled HDA–HFBA derivative used as the internal standard. Column: J & W Scientific fused-silica column coated with DB-5 bonded stationary phase (30 m × 0.247 mm I.D.), film thickness 0.25 μm. Inlet pressure of the carrier gas (helium): 1.0 kg/cm^2. Splitless injection (1 min) of 4 μl of toluene. Temperature programming: isothermal at 100°C (2 min), increased at 30°C/min to a final temperature of 280°C, which was maintained for 1 min.

However, when using CI with ammonia as the reagent gas, the M + 18 ions of HDA–HFBA and tetradeuterated HDA–HFBA (*m/z* 526 and 530, respectively) were the most abundant. The abundance of the M + 1 ions (*m/z* = 509 and 513, respectively) was less than 2%. Using isobutane as the reagent gas, the M + 1 ions were the most abundant (*m/z* 509 and 513, respectively). The relative abundances of the M + 18 ions were *ca.* 60%.

SIM with ammonia as reagent gas (monitoring the ions of *m/z* 526 and 530, respectively) showed a *ca.* ten times higher sensitivity than that with isobutane (*m/z* = 509 and 513, respectively). Also, the selectivity when analysing urine samples was improved. The contamination of the ion source was much lower when ammonia was used. The analysis of several hundred of samples without cleaning the ion source were therefore possible. Baseline separation of the investigated compounds relatively the urine matrix was also found. The use of ammonia as reagent gas was therfore concluded to be the best choice for the determination of HDA in hydrolysed urine.

Chromatography

The chromatographic behaviour of the amide derivatives was excellent. The use of a column with an apolar stationary phase with relatively low film thickness was preferred owing to the lower temperature and column bleeding. Chromatograms of urine samples originating from HDI-exposed subjects are shown in Fig. 2. No interfering peaks disturb the evaluation of the chromatogram using SIM and CI.

Quantitative analysis

Recovery. On extracting HDA from aqueous solutions by the addition of saturated NaOH to an organic phase, no losses were found. However, on extracting urine hydrolysates by the same procedure, considerable losses were found. On spiking human urine and performing the work-up procedure, the overall recovery was found to be 34 ± 4% (n = 12) for a concentration of 100 μg of HDA per litre of urine.

Calibration graphs. Human urine was spiked by adding different amounts of HDA and performing the work-up procedure as described above. For each HDA concentration two determinations, with duplicate injections, were made. No significant difference between plotted peak heights or peak-areas ratios, relative to the internal standard, was observed. The investigated concentration range of 1–30 μg/l in urine gave a correlation coefficient of 0.985 for the peak-area ratio measurements (n = 7).

Precision. The overall precision was found to be 5% (n = 9) for human urine spiked with 22 μg/l of HDA.

Detection limit. The detection limit using SIM and EI was set by the matrix and was found to be more than 100 μg/l of HDA in urine.

The detection limit using SIM and CI with ammonia as reagent gas was *ca* 0.5 μg/l of HDA in urine. No interfering peaks appeared when urine samples from five unexpected subject were examined. As the detection limit is set by the instrument, it would be possible to lower the detection limits by using a more sensitive instrument and by further enrichment of the sample.

Application

A male subject was exposed to HDI for 7.5 h in a test chamber. The average

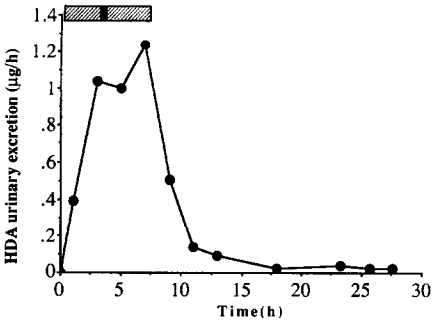

Fig. 3. Urinary excretion of HDA and hydrolysable HDA conjugates for a subject exposed to HDI for 7.5 h in a test chamber. The HDI air concentration was *ca.* 30 $\mu g/m^3$ and the estimated inhaled dose of HDI was *ca.* 100 μg. Urinary excretion is shown at the mid-time of each observation period. Bar: shaded area, HDI exposure; black area, exposure-free period.

concentration of HDI was *ca.* 30 $\mu g/m^3$, which corresponds to *ca.* 85% of the threshold limit value (TLV) in Sweden (35 $\mu g/m^3$). The estimated dose of inhaled HDI was *ca.* 100 μg. All urine was sampled before, during and after the exposure, and the related amine HDA was determined in hydrolysed urine. The half-time of urinary levels of HDA was short ($t_{1/2} \approx 1.4$ h) and >90% of the urinary elimination was completed within *ca.* 4 h after termination of the exposure (Fig. 3). The detection limit of the method was sufficient for the determination of HDA in urine, thus offering the possibility of biological monitoring of occupational HDI exposure.

CONCLUSIONS

A method has been developed for assessing occupational exposure to HDI and HDA. Selective and sensitive determination of HDA in hydrolysed urine, at low $\mu g/l$ levels, was possible using GC–MS. The use of tetradeuterated HDA as internal standard was demonstrated to give accurate and precise determinations. The method makes it possible to determine HDA in urine of subjects exposed to HDI at the Swedish TLV level.

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