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CHROMATOGRAPHIC DETERMINATION OF AMINES IN BIOLOGICAL FLUIDS WITH SPECIAL REFERENCE TO THE BIOLOGICAL MONITORING OF ISOCYANATES AND AMINES

I. DETERMINATION OF 1,6-HEXAMETHYLENEDIAMINE USING GLASS CAPILLARY GAS CHROMATOGRAPHY AND THERMIONIC SPECIFIC DETECTION

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SUMMARY

A capillary gas chromatographic method with thermionic specific detection was developed for the analysis of 1,6-hexamethylenediamine (HDA) (*C.A.* No. 124-09-4) in aqueous solution, human urine and plasma. The method is based on a derivatization procedure with ethyl chloroformate (*C.A.* No. 541-41-3) in a two-phase system, where HDA-diurethane is formed. The overall recovery was found to be 95 ± 5% for a concentration of 100 μg HDA/l of urine, 98 ± 5% for 1000 μg/l. In plasma the recovery was 106 ± 4% for 500 μg/l. The minimum detectable concentration in plasma and urine was found to be less than 10 μg/l. The use of heptafluorobutyric anhydride as a derivatization reagent is also discussed.

INTRODUCTION

Aliphatic diamines are industrially used as chelating agents and chemical reagents. 1,6-Hexamethylenediamine (HDA) is used for the manufacture of urethane coatings and of polyamides, *e.g.* Nylon-66, also in paints and as a curing agent for epoxy resins. HDA is moderately toxic[1–3], and has been associated with health hazards in the work environment[4,5].

Analogous endogenous diamines, *e.g.*, putrescine (1,4-diaminobutane) and cadaverine (1,5- diaminopentane), are known to have important roles in cell growth and differentiation. HDA has been identified in humans, as one in a series of metabolites of the cell-differentiating agent hexamethylenebisacetamide (HMBA)[6–9]. Rosenberg and Savolainen[10] have proposed that analysis of HDA in urine may be used as a test for occupational exposure to 1,6-hexamethylenediisocyanate (HDI).

The occurrence of diamines in working atmospheres, and the biochemical interest in di- and polyamines, has necessitated development of a series of analytical methods, including high-performance liquid chromatography (HPLC)[11–14], gas

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chromatography (GC)[15], ion-exchange and ion-pair chromatography[16,17] and thin-layer chromatography[18]. Monitoring of HDI in air using impinger flasks containing acidic aqueous solutions, where HDA is formed, was described by Dalene[15]. The amine was derivatized to the corresponding amide by reaction with a perfluoro fatty acid anhydride. Capillary GC with electron-capture (ECD) and thermionic specific detection (TSD) was used for quantitation.

A possibility for biological monitoring of exposure to HDI and HDA would be valuable, and the aim of this study was to develop an highly sensitive analytical method for the determination of HDA in biological fluids.

EXPERIMENTAL

Apparatus

A Varian 3500 gas chromatograph equipped with a Varian thermionic specific detector and a Varian 8035 automatic on-column injector was employed. The injector was cooled with liquid nitrogen. The injector starting temperature was 105°C for 5 s, and thereafter the temperature was increased with 150°C/min to a final temperature of 280°C, where it was kept for 7 min. Column parameters as in Fig. 2.

A Varian 3700 gas chromatograph equipped with a Varian thermionic specific detector and a Grob-type cold on-column injector with a water cooling system, designed and manufactured at our laboratories, was also used. Typical settings for the detector were: bead heating current, 3.10 A; bias voltage, − 3.9 V, detector temperature, 270°C; gas flow-rates; 3 ml/min of hydrogen, 175 ml/min of air and 20 ml/min of nitrogen as the make-up gas.

A Shimadzu GC–MS QP1000 EI/CI quadrupole mass spectrometer connected to a Shimadzu GC 9A gas chromatograph was used.

Chromatograms were recorded on Servogor Model 310 linear recorders, and Shimadzu C-R3A integrators were used for peak evaluation. An Heidolph VV 2001 Rota-vapor, rotating evaporator (Heidolph Elektro, Kelheim, F.R.G.) connected to an aspiration pump was used for evaporation, and a Sigma 3E-1 centrifuge (Sigma, Harz, F.R.G.) was employed for phase separation.

A Reacti-Vap™ Evaporator (Pierce) was used for evaporation of toluene sample solutions containing the carbamate derivative of HDA.

For enrichment and evaporation of solvent containing heptafluorobutyric anhydride (HFBA) derivatives, a vacuum desiccator connected to an aspirating pump was used. The apparatus was supplied with an electrically heated oven, designed and manufactured at our laboratory.

Columns

Fused-silica capillary columns with chemically bonded stationary phases, CP-Sil® 8 CB (Chrompack, Middelburg, The Netherlands), 12 m × 0.32 mm I.D., with a film thickness of 0.2 μm, and DB-5 (J & W Scientific, Folsom, CA, U.S.A.), 30 m × 0.24 mm I.D., with a film thickness of 0.5 μm were used.

Chemicals

Chemicals used were HDA, ethyl chloroformate, isobutyl chloroformate and toluene from Janssen (Beerse, Belgium). Trifluoroacetic anhydride (TFAA), penta-

fluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) were from Pierce (Rockford, IL, U.S.A.), di-*n*-butylamine (DBA) and 1,4-butanediamine (BDA) from Fluka (Buchs, Switzerland), HCl, NaOH, NH_3 and K_2HPO_4 from Merck (Darmstadt, F.R.G.).

Standards

Synthesis of 1,6-hexamethylenediurethane and 1,4-butanediurethane. To 250 ml of a solution of toluene containing *ca.* 5 g of HDA or BDA were added 25 ml of 30% (w/w) ammonia and 5 g of sodium hydroxide dissolved in 250 ml of distilled water. Ethyl chloroformate (20 ml) was added dropwise with stirring, and the mixture was stirred for 10 min. The organic layer was separated and shaken four times with 200-ml portions of 0.1 *M* HCl, to remove unreacted amine and partially reacted amino-urethane. The toluene solution was then washed four times with 200-ml portions of water, and evaporated to dryness to remove the remaining ethyl chloroformate reagent. After recrystallization from ethyl acetate the derivatives were stored in an desiccator.

Standards and internal standards were identified by GC–mass spectrometry (MS), and the purity was tested using TSD and flame ionization detection (FID). The purity was also examined by elemental analysis, and was found to be better than 99%.

Synthesis of the isobutyl chloroformate derivative of DBA. To 250 ml of a toluene solution containing *ca.* 5 g of DBA, 25 ml of 30% (w/w) ammonia and 5 g of sodium hydroxide, dissolved in 250 ml of distilled water, were added. Isobutyl chloroformate was added dropwise to the solution with continuous stirring, and the mixture was stirred for 10 min. The organic layer was separated and shaken four times with 200-ml portions of 0.1 *M* HCl to remove unreacted amine. The toluene solution was then washed four times with 200-ml portions of water, and evaporated to dryness to remove the remaining ethyl chloroformate reagent. The residue was redissolved in ethyl acetate and eluted through a silica column (60 mm × 20 mm I.D.). The recrystallization from ethyl acetate was performed at −10°C.

Synthesis of 1,6-hexamethylenebisheptafluorobutyramide. 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 to 150°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. The organic phase was eluted through a silica column (60 mm × 20 mm I.D.) with ethyl acetate, followed by evaporation of the ethyl acetate solution to dryness; *ca.* 500 mg (80%) of the amine were obtained.

Procedure

Preparation of standard solutions. Standard solutions of the derivatives of HDA were prepared by dissolving accurately weighed amounts of each derivative (*ca.* 60 mg/100 ml) in toluene. The solutions were then further diluted in toluene to appropriate concentrations. Standard solutions of HDA were prepared in acidic-aqueous solutions.

Sampling and storing of urine samples. Urine samples were acidified by the addition of 5 ml of aqueous 6 *M* HCl solution per *ca.* 100 ml of urine. The urine samples were stored in a refrigerator.

Work-up procedure

With chloroformates as the derivatization agents. A 2-ml urine or plasma sample was added to 3 ml of 6 *M* HCl. The mixture was heated at 100°C overnigh (hydrolysis). A 2-ml aliquot was transferred to a test-tube where 1 ml of 3% (w/w) aqueous NH_3, 3 ml of 5 *M* NaOH and 2 ml of toluene were added. The mixture was shaken and 100 μl of ethylchloroformate were added. The mixture was then shaken for 15 min at room temperature. A 1-ml aliquot of the toluene layer was evaporated to dryness by using a flow of nitrogen (30°C). A 1-ml volume of a toluene solution, containing an internal standard comprising the isobutyl chloroformate derivative of di-*n*-butylamine at the appropriate concentration, was added to the dry residue. The toluene layer was analysed by GC. The same procedure was used for aqueous samples where the hydrolysis step can be eliminated.

With anhydrides as the derivatization agents. A 2-ml urine sample was added to 3 ml of 6 *M* HCl. The mixture was heated at 100°C overnight. A 2-ml aliquot was transferred to a test-tube where 4 ml saturated NaOH and 3 ml toluene were added. The mixture was shaken for 5 min at room temperature. A 2-ml volume of the organic layer was transferred to a new 10-ml test-tube where 20 μl of HFBA were added. The solution was immediately shaken vigorously for 5 min on a Vortex mixer. The excess of HFBA and the acid formed were removed by shaking with 2 ml of 1 *M* phosphate buffer solution (pH 7.5). The toluene layer containing the amide formed was then transferred to a new test-tube, ready for injection on the chromatographic system. The same procedure was used for aqueous samples where the hydrolysis step can be eliminated.

Internal standard. The internal standards used were 1,4-butane diurethane, the isobutyl chloroformate derivative of di-*n*-butylamine (1 ng/μl) and the HFBA derivative of 1,4-butanediamine (1 ng/μl). A solution of toluene containing the internal standard was added to the dry residue from the work-up procedure before the GC analysis.

RESULTS AND DISCUSSION

Storage and treatment of samples

HDA-spiked urine samples were found to be stable after the acidification. No noticeable change in sample composition occurred even after storage in darkness at room temperature for several weeks.

The mixture was heated at 100°C overnight in order to hydrolyse the sample; a shorter time of hydrolysis was shown to result in lower recovery of HDA. However urinary elimination of compounds originating from exposure to HDI or HDA will rise a mixture of several metabolites and conjugates, of which free HDA probably consitutes a minor portion. The hydrolysis reaction obviously influences the possibility of separately determining these compounds, and consequently the method described only gives the sum of free HDA and hydrolysable HDA conjugates. Sample clean-up using a silica gel cartridge has been described as efficient[10]. However, our attempts to perform this and other similar procedures were unsuccessful due to severe losses (*ca.* 50%).

Work-up procedure

Chloroformates as reagents. Parameters for the derivatization of amines have

been studied in some detail[19]. In general, the derivatization reaction in a two-phase system, with an appropriate pH in the aqueous phase, will be faster than that in a single-phase system. The choice of pH depends on the basicity of the amine considered. For the reaction to occur at a reasonable rate at room temperature unprotonated amines must be present. For the two-phase derivatization of piperazine[19] (pK_a = 9.8) we found that with a phosphate buffer solution (pH 10) the reaction is quantitative in less than 5 min, with ammonia as a catalyst. However, this procedure gave unsatisfactory results for the very basic diamine HDA (pK_a = 11.9), whereas the reaction is quantitative only at pH > 12. The recovery for two-phase derivatization of HDA in urine (*ca.* 2 ng/μl) is quantitative, for ammonia concentrations higher than 1% (w/w) (Fig. 1). We found that the use of 3% ammonia is suitable, since the ammonia concentration in water solutions tend to decrease during storage. The amount of ethyl chloroformate added is not critical when using ammonia. The plot of the amount of ethyl chloroformate added *versus* recovery in Fig. 1 is virtually horizontal in the range 10–150 μl. It was also observed, for the system without ammonia, that the recovery decreased when the amount of ethyl chloroformate added was increased.

It should also be noted that the consumption of the reagent due to hydrolysis in the standard procedure is not critical if the reagent is added directly to the two phase system. This was established by adding the HDA-containing urine hydrolysate to the two-phase reaction mixture at different times. The same quantitative results were found for 0–1 min, whereafter the recovery decreased, and after 3 min only *ca.* 50% recovery was obtained. Injection of chloroformate reagents influences the detector sensitivity as described previously[19]. The evaporation step is essential to remove excess of reagent. The procedure is time consuming, when a Rota-vapor is used, as only a single sample can be evaporated. Evaporation by a flow of nitrogen simplifies routine analyses as nine samples can be treated simultaneously.

Anhydrides as reagents. The two-phase derivatization of aromatic diamines was recently studied[20]. However the two-phase derivatization procedure was originally

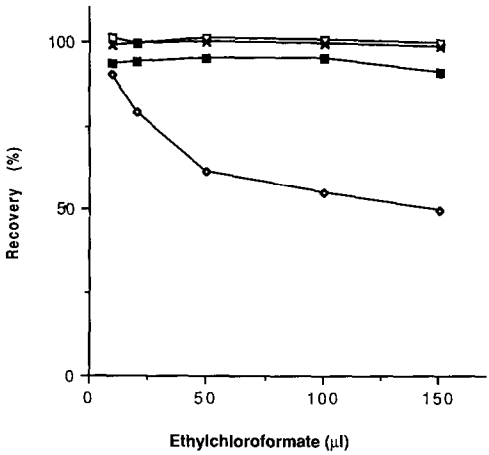

Fig. 1. The recovery of HDA-diurethane with variable amounts of the derivatization reagent (ethyl chloroformate) and different concentrations of ammonia: 0.0 (◇), 0.5 (■), 1.0 (□) and 5.0% (×).

developed for aqueous solutions, and has not yet successfully been used for urine samples containing aliphatic diamines. For urine samples the recoveries were low (less than 50%), and varied greatly, giving poor reproducibility. The derivatization procedure was therefore performed in toluene after extraction of aqueous urine hydrolysates.

The excess of reagent was efficiently removed from the organic phase by extraction with a 1 *M* phosphate buffer (pH 7.5) solution. For urine samples, using GC–TSD, the chromatograms of the derivatives showed a more complex picture compared to the corresponding chloroformate derivatives. Further work-up by liquid extraction, membrane extraction or by the use of extraction columns may be necessary.

Chromatography

The chromatographic behaviour of both the urethane and amide derivatives is excellent. The use of an apolar stationary phase with relatively low film thickness is

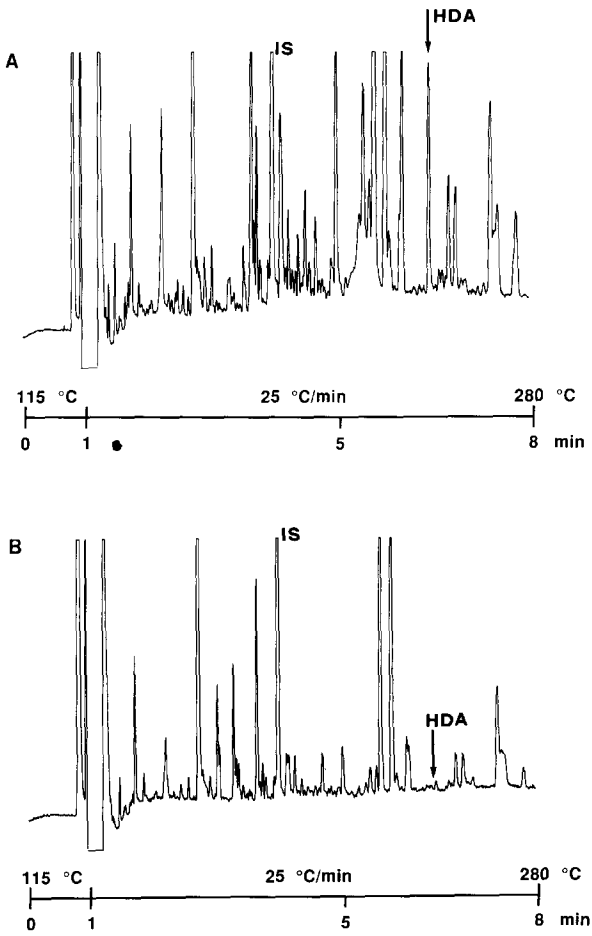

Fig. 2.

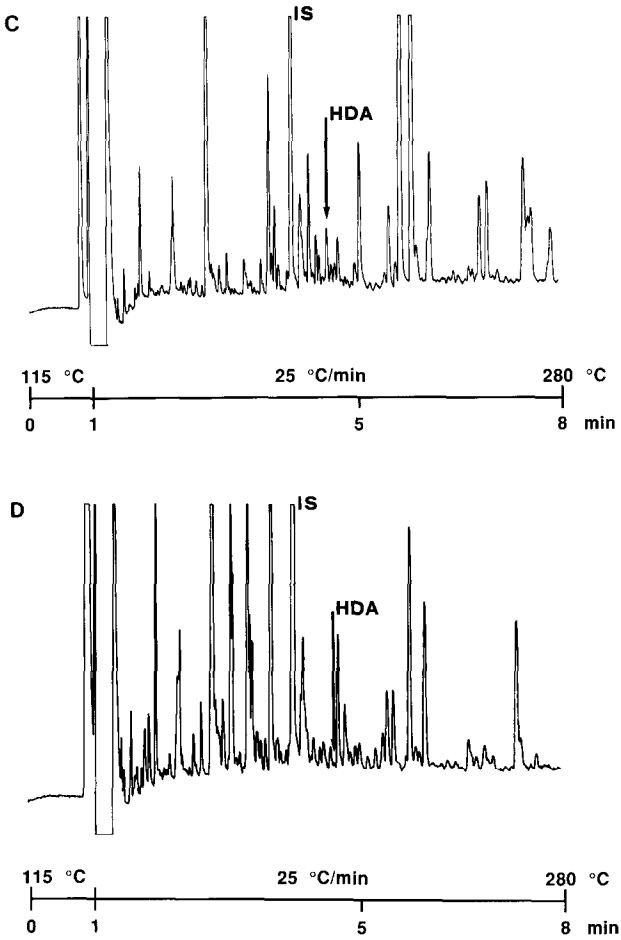

Fig. 2. Chromatograms of HDA at *ca.* 200 μg/l in hydrolysed urine treated with ethyl chloroformate (A) and HFBA (C) using TSD. Corresponding blank traces were for ethyl chloroformate (B) and for HFBA in urine (D). All chromatograms were performed using GC–TSD with on-column injection of 1 μl toluene solution. Internal standard (IS): the isobutyl chloroformate derivative of di-*n*-butylamine at 1 ng/μl. Capillary column: J&W fused silica coated with DB-5 bonded stationary phase (30 m × 0.24 mm I.D.), film thickness 0.5 μm. Temperature programming as shown. Carrier gas (helium) at 2.3 kg/cm^2. Thermionic specific detector: bead heating current, 3 A; bias voltage, −3.5 V; temperature 280°C. Flow-rates: hydrogen, 4; air,180; make-up (helium), 10 ml/min.

preferred, due to the fact the HDA-diurethane can decompose to HDI at elevated temperatures. Analysis of the HDA-diurethane by GC–MS showed that this compound actually decomposed into HDI. The HDI appeared as a tailing peak earlier in the chromatogram, and increased with increasing elution temperature. Due to the decomposition at elevated temperatures, higher film thicknesses should be avoided, and when using film thicknesses less than 0.8 μm no problems occurred. Chromatograms of urine samples, originating from an human oral administration of HDA, are

shown in Fig. 2, and for urine matrixes it is evident that the chloroformate procedure is a better choice than the anhydride procedure. No interfering peaks disturb the evaluation of the chromatogram.

Quantitative analysis

Recovery. The recovery was studied by spiking aqueous solutions, hydrolysed human urine and blood plasma, and performing the derivatization procedures as described above. Peak heights were compared to those of standards by using GC–TSD. With ethyl chloroformate as the reagent, the recovery for human urine was 95 ± 5% ($n=7$) for a concentration of 100 μg of HDA per litre and 98 ± 5% ($n=7$) for 1000 μg/l and for plasma 106 ± 4% ($n=6$) for 500 μg/l at a 95% degree of confidence.

Calibration graphs. Fig. 3 shows the calibration graph for HDA derivatives of ethyl chloroformate obtained by GC–TSD. Different amounts of HDA were added to urine and the work-up procedure was as described above. For each HDA concentration, two determinations with duplicate injections were made. No significant difference between plotted peak heights or peak area ratios, relative to the internal standard, was observed. The concentration range investigated, 10–350 μg/l urine, gave a correlation coefficient of 0.9998 for a plot of the area ratio for six concentrations. No noticeable differences were found between aqueous solutions and human urine.

Precision. When analysing substances present in complicated matrices such as urine and plasma, the addition of an internal standard to the sample before the analysis is strongly recommended. In this study, the internal standard chosen behaves similarly to the HDA derivatives in the chromatographic system. The overall precision, using the ethyl chloroformate work-up procedure and the di-*n*-butylamine derivative as the internal standard, was very good. The overall precision with the work-up procedure and GC analysis was about 6% ($n=6$) for 100 μg/l HDA-spiked urine, and 2% ($n=5$) for 1000 μg/l with duplicate samples and injections. The overall precision (R.S.D.) for urine samples was 5% (n = 8) at a concentration of 100 μg/l, and 3% at a concentration of 1000 μg/l.

Detection limit. Using GC–TSD the responses for the derivatives of HDA investigated were in the same range. In acidic aqueous air sampling solutions, where the chromatograms appear to be virtually free from interfering peaks, the detection limits were less than 10 pg injected amount, which corrsponds to a concentration of 10

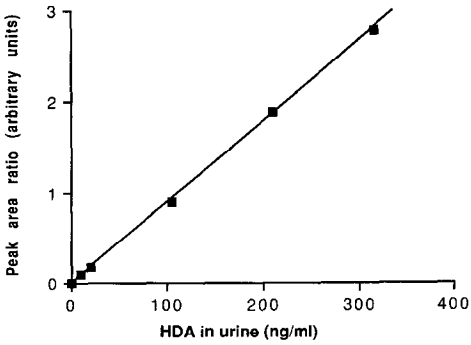

Fig. 3. Calibration graph for HDA in humane urine.

μg/l of sampling solution. The same detection limit was found for the ethyl chloroformate derivative in biological fluids. The detection limit can be further lowered by dissolving the dry residue in a smaller volume, and an enrichment factor of up to 50 is possible. The detection limit for the HFBA derivatization procedure, when used for biological samples such as urine, is considerable higher due to the interfering peaks (Fig. 2).

CONCLULIONS

The chromatographic behaviour of both the urethane and amide derivatives is excellent, and the choice of reagent is principally affected by the matrix and by the detection system used. For urine hydrolysates, the two-phase derivatization with ethyl chloroformate is preferable as the work-up procedure is performed in one step. The method presented offers enhanced possibilities to determine endogenous and exogenous aliphatic diamines at low concentrations (μg/l range) in biological fluids. The method is also applicable to the determination of HDI in air using the impinger technique and an acidic aqueous sampling solution.

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