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Note

Detection of urinary amine metabolites in toluene diisocyanate exposed rats

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Toluenediisocyanate is one of the principal monomers used in the production of flexible polyurethane foams, elastomers, coatings or adhesives[1]. The commercially available product usually contains 80% of the 2,4- and 20% of the 2,6-diisocyanate isomer[2]. The isocyanate moiety reacts readily with molecules capable of a hydrogen exchange resulting in, *e.g.*, the urethane bond, or if the reactant is water the reaction yields carbon dioxide and the corresponding amine. Because of this high inherent reactivity organic isocyanates are notorious skin irritants and allergic sensitizers[3,4].

The isocyanates occur in the atmosphere of workplaces where polyurethane articles are produced. The low vapour pressure of most monomer molecules indicates that the amount of vapour must be small while the aerosol fraction may be significant owing to the use of spray guns in production technology. Another important mode of exposure is by percutaneous absorption as the degree of polymerization is often tested by the workers with bare hands or finger tips. Monomer spills on clothing and exposed skin areas are also common.

To verify the postulated percutaneous exposure and to develop a biological exposure test we carried out animal experiments with a recently validated technique[5]. No original isocyanate was detected in the urine collected after the exposure while conjugated toluenediamine was found by two chromatographic techniques and identified by mass spectrometry. The method is clearly more sensitive than earlier liquid chromatographic techniques and is easier to apply in practice than an earlier blood test with a very critical timing, because of the short half-life of the organic amines in the circulation.

EXPERIMENTAL

Materials

Toluene-2,4-diisocyanate was obtained from Fluka (Buchs, Switzerland), heptafluorbutyric anhydride (HFBA) from Pierce Chemicals (Rockford, IL, U.S.A.) and di-*n*-butyl ether from BDH Chemicals (Poole, U.K.). All other reagents were of analytical reagent grade unless otherwise stated.

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Exposure conditions

Skin tissue of rats was exposed to 40% toluene-2,4-diisocyanate solution in di-*n*-butyl ether liquid[3] by a technique developed for the evaluation of local neurotoxicity of organic solvents[5]. The rats were exposed for three hours daily on four successive days. Urine was collected in metabolic cages for 18 h after the fourth exposure. The samples were stored at −25°C until the analysis.

Urine sample preparation

The urine aliquots (1 ml) were made alkaline by adding saturated sodium hydroxide solution (2 ml). The free amines were extracted into toluene (1 ml), and the organic phase was removed after centrifugation. Sodium chloride (0.5 g) was added before extraction for better phase separation. Acid back-wash was used for cleanup of the organic phase, then 0.1 *N* sulphuric acid (1 ml) was added to the toluene layer, and, after shaking for 2 min, the organic phase was discarded. The amine was recovered from the water layer by alkaline re-extraction with toluene (1 ml) and saturated sodium hydroxide solution (2 ml). The toluene layer was removed and dried over sodium sulphate.

For the determination of the total amine (free and conjugated) concentration, 1 ml of urine was submitted to hot acid hydrolysis in 6 *N* hydrochlorid acid (1 ml) at 100°C for 45 min. The hydrolysate was cooled to room temperature and the liberated amine recovered as described above.

Formation of heptafluoramide derivative

One ml of the toluene extract was mixed with 20 μl of HFBA. Excess of HFBA was removed after 10 min by extraction with 1 ml of 1 *M* dihydrogen phosphate buffer (pH 7). The toluene layer containing the amine derivative was removed and dried over sodium sulphate.

Gas chromatographic techniques

A Varian Model 3700 gas chromatograph equipped with a packed column injector and a Varian Thermionic Specific detector was used for the nitrogen-selective detection of the underivatized amines. The detector was operated at 250°C with a bead heating current of 7.1 A and bias voltage of −5 V, and hydrogen (4.5 ml/min) and air (175 ml/min) were passed through the detector. The injector temperature was 220°C, and nitrogen was used as carrier gas (30 ml/min). A glass column (0.5 m × 1.2 mm I.D.) with 28% Pennwalt amine packing with 4% potassium hydroxide on Gas-Chrom R (80–100 mesh) (Applied Science, Deerfield, IL, U.S.A.) was used. The column was operated at 200°C. The minimum detectable concentration was 0.1 μg/ml, which corresponded to 100 pg with a 1-μl injection volume. The retention time of toluene-2,4-diamine was 4.0 min.

A Varian Aerograph Series 1400 gas chromatograph equipped with a ^{63}Ni electron-capture detector and an autolinear temperature programmer was used for the analysis of the derivatized amine. The original packed column mode of the chromatograph was changed to capillary version by installing a home-made Grob-type split/splitless injector with an I.D. of 3 mm and a volume of 0.85 ml. Nitrogen was used as make-up gas (20 ml/min). The injector temperature was 220°C and was operated in the splitless mode (30 sec). Fused-silica capillary columns SE-52 (30 m ×

0.26 mm I.D.; 0.25-μm film thickness) (J&W Scientific, Rancho Cordova, CA, U.S.A.) and OV-1701 (25 m × 0.32 mm I.D.; 0.15-μm film thickness) (Orion Analytica, Espoo, Finland) were used. Nitrogen was used as carrier gas (2.0 ml/min) and a typical temeprature programme was 3 min at 110°C followed by heating to 230°C at 6°C/min and maintaining the final temperature for 1 min. The minimum detectable amount was 10 pg with a 1-μl injection. The retention time of the HFBA derivative was 13 min.

Gas chromatography–mass spectrometry

The identity of the amine was confirmed by gas chromatography–mass spectrometry of the derivatized compound. The mass spectrometer consisted of a Hewlett-Packard (5990A) electron ionization (70 eV) quadrupole mass-selective detector equipped with a 5990A Series Hewlett-Packard gas chromatograph with an on-column injector. A SE-54 fused-silica capillary column (20 m × 0.32 μm I.D.; 0.15-μm film thickness) (Orion Analytica, Espoo, Finland) was used with helium as a carrier gas (1 ml/min). The injector temperature was 50°C and the column programme was 0.5 min at 70°C and heating to 250°C at 16°C/min. The HFBA derivative of toluene-2,4-diamine was retained for 6.6 min. The detector was operated at 2000 V.

Recovery studies

A standard curve for the calculation of the toluene-2,4-diamine concentrations in the urine was obtained by adding variable amounts of toluene-2,4-diamine (0.05–1.2 μg/ml) to a blank sample of urine which, was analysed together with the actual specimens.

RESULTS AND DISCUSSION

A linear correlation was found between toluene-2,4-diamine added to the urine and peak height (Fig. 1). The recovery of the added toluene-2,4-diamine from the unhydrolyzed and from the hydrolyzed urine was 85 ± 4% (± S.D.; n = 9) and 70 ± 5%, respectively.

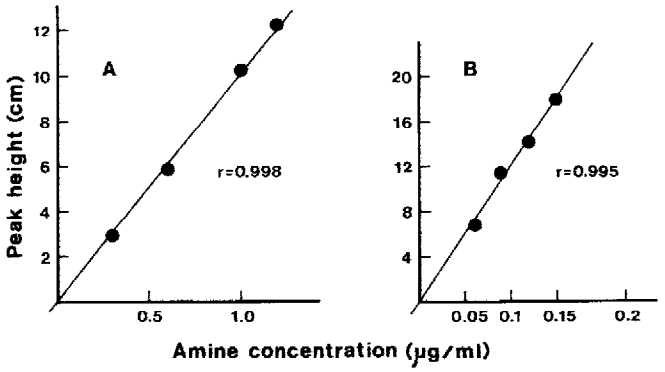

Fig. 1. Standard curves of toluene-2,4-diamine. (A) As free base with nitrogen-selective detection and (B) as HFBA derivative with electron-capture detection. Note that the derivatization yields a 10-time greater sensitivity.

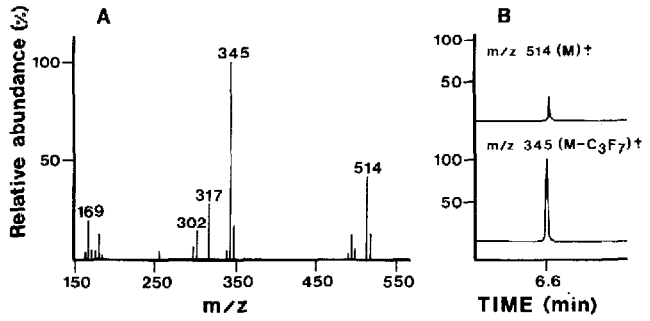

Fig. 2. (A) Mass spectra of HFBA derivative of toluene-2,4-diamine standard and (B) selected-ion monitoring of toluene-2,4-diamine from urine sample. The ions correlate to the following presumed fragmentation: m/z 514 $(M)^{\ddagger}$; 345 $(M-C_3F_7)^{\ddagger}$; 317 $(M-C_3F_7CO)^{\ddagger}$; 302 $(M-C_3F_7CONH)^{\ddagger}$; and 169 $(C_3F_7)^{\ddagger}$. Time scale (B) indicates retention in the gas chromatograph.

No free toluene-2,4-diamine was detected in the urine of rats exposed to the isocyanate. The concentration of toluene-2,4-diamine in hydrolyzed urine was 1.5 μg/ml (range = 1.3–1.6 μg/ml; n = 5), corresponding to 12.5 μmol/l (10.7–13.1 μmol/l). Gas chromatography–mass spectrometry confirmed the identity of the amine (Fig. 2). Representative gas chromatograms of urinary toluene-2,4-diamine before and after acid hydrolysis are shown in Fig. 3.

Several methods for the determination of amines in metabolic studies in the concentration range of nmol/ml have been reported in the literature, employing both liquid and gas chromatography[6–8]. However, trace assay of amines is possible only with the highly sensitive electron-capture detector described above. Furthermore, the extraction of the amines from the aqueous medium depends strongly on the alkalinity. It has been shown in experimental detail[9] that very high hydroxyl ion concentrations should be used.

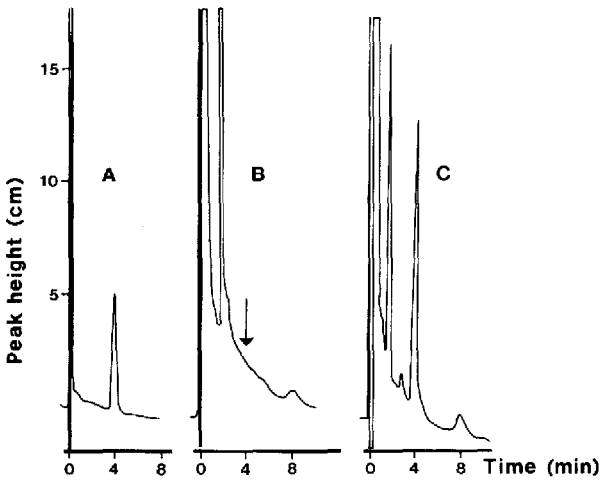

Fig. 3. Gas chromatograms with nitrogen-selective detection of (A) 0.48 μg/ml of toluene-2,4-diamine standard (4 min) and from the urine of exposed rats (B) unhydrolyzed urine and (C) hydrolyzed urine, toluene-2,4-diamine 1.6 μg/ml (4 min). The B configuration is identical to control urine. The arrow (B) indicates the retention of the possible free diamine.

It is noteworthy that we could not demonstrate free toluene-2,4-diamine in the urine of the exposed rats. For that matter, no free toluene-2,4-diisocyanate was found, either. The latter is understandable in view of the great reactivity of the organic isocyanate groups. It is very likely that a major part of isocyanate had already reacted with water in the skin and with the skin-cell macromolecules allowing only absorption of the corresponding amines.

Rat tail has many advantages as a model for the percutanous exposure. It is virtually hairless and it has a lively circulation[10] so that the absorption of epicutaneous chemicals is favoured, because the steady states may not be reached due to the rapid removal of the molecules in the interstitium by the rapid blood flow. The hornified skin of the rat tail is thicker than human skin. Therefore, it is likely that the toluene-2,4-diisocyanate spilled on human skin is even more actively absorbed.

Organic primary amines are typically acetylated for their excretion in the urine[11]. It is conceivable that in a massive exposure this metabolic pathway may be saturated, which would explain the earlier report of free amines in plasma and urine of exposed rats[12]. For comparison, another organic diamine, 4,4′-diaminodiphenylmethane, is rapidly removed from the circulation of experimentally exposed rats[13]. However, this may not show its rapid excretion, but a rapid N-acetylation in liver. This also renders the blood-amine determinations difficult as the time of the sampling seems to be so critical. The urine tests are often acceptable in the clinical practice as no venipuncture is needed. It also has the advantage that urine in the bladder provides an indication of the accumulated dose over time rather than the concentration of the chemical in the transmitting blood compartment.

Our preliminary experience indicates that the analytical techniques are suitable also for other amines and may be used for the detection of occupational exposure. This is especially facilitated by the employment of mass spectroscopy in the identification of the isolated amines.

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