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Note

Gas chromatographic identification of urinary metabolites of insect repellent N,N-diethylphenylacetamide on inhalation exposure in rats

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N,N-Diethylphenylacetamide (DEPA) is a new cost-effective and pluri-potent insect repellent [1–4]. So far, the effects of DEPA on laboratory animals have been examined by dermal application [3,5–8] and by oral administration [5,7,9]. Recently it was reported [8] that N-ethylphenylacetamide (EPA) and phenylacetic acid (PhAA) were excreted in the urine of rabbits following dermal application of DEPA. The toxicity and metabolism of DEPA on inhalation are not known, though this is also an important route of exposure since insect repellents are also used as aerosols.

This paper reports an investigation into the metabolic fate of DEPA that enters into living systems by inhalation. It was observed that unmetabolized DEPA and phenylacetamide (PA) are also excreted when rats are exposed to the chemical.

EXPERIMENTAL

Chemicals

DEPA, EPA and PA were synthesized by methods described previously [2,8]. PhAA and benzoic acid (BA) were procured from Fluka (Buchs, Switzerland). Methanol, chloroform and sodium sulphate of analytical-reagent grade were procured from Merck (India) and Sarabhai (India).

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Inhalation experiments

Whole body exposure to DEPA aerosol. Nine young male albino rats (Wistar strain) weighing 71 ± 8.5 g were exposed to DEPA in two batches. Aerosols of DEPA were generated using an air-blast glass nebulizer. The animals were exposed dynamically in individual stainless-steel cages in an all-glass whole body exposure chamber with a volume of 21 l. The concentration of DEPA in the chamber atmosphere was determined as follows. The chamber air (570 ml) was trapped in a round-bottomed flask, and 2 ml of ethyl acetate were injected into the flask through a PTFE adapter with a silicon diaphragm and thoroughly mixed. The ethyl acetate solution was analysed for DEPA concentration by gas chromatography (GC) [3]. The concentration was adjusted to 1450 mg/m^3, and the animals were exposed for 4 h (1 LC50, R. Vijayaraghavan, unpublished results). The particle size was determined using a Royco particle monitor (Royco, U.S.A.) and was found to be within the respirable range (<3.0 μm). The t_{99} was 4.6 min.

The urine samples were collected directly from the bladders of rats sacrificed after exposure for immediate processing in three pools for identification of DEPA metabolites by GC.

Head only exposure to DEPA aerosol. Twelve young male albino rats weighing 70 ± 10 g were divided into two groups of six each. One group served as the control. Rats of the second group were exposed to aerosols of DEPA (1160 mg/m^3; 0.8 LC50[a]) for 4 h in the manner described earlier, with a difference in the mode of exposure only. Their heads were exposed to the chamber atmosphere while they were held in special individual adapters connected horizontally at right angles to the axis of main body of the inhalation chamber. These adapters were designed to minimize possible cutaneous absorption of DEPA, which is a lipophilic compound. Urine samples (24 h) were collected with chloroform as preservative in clean cooled glassware from control and from DEPA-exposed rats.

Sample work-up

Aliquots of the samples were extracted in two steps with chloroform–methanol (2:1), essentially following the method of Surina et al. [10]. In the first step, amide metabolites of DEPA were extracted at pH 7. In the second step, the aqueous phase from step 1 was subjected to alkaline hydrolysis for 2 h followed by chloroform–methanol extraction at pH 2 for the recovery of organic acids released from conjugates by the hydrolytic procedure. Organic phases obtained in the two steps were separately dried over anhydrous sodium sulphate and concentrated for GC analysis.

GC analysis on 15% diethylene glycol succinate (DEGS) stationary phase

Samples obtained in the first set of whole body exposure experiments were processed and analysed by the following method. A Nucon gas chromatograph

[a]LC50 is the concentration lethal to 50% of the animals for the said duration of exposure (4 h). The animals were observed for mortality till seven days.

(Nucon Engineers, New Delhi, India) equipped with a hydrogen flame ionization detector and a strip chart recorder (Digital Electronics, Bombay, India) was used in the study. A stainless-steel column (1.8 m×3 mm O.D.) packed with 15% stabilized DEGS on Chromosorb W HP was used.

The analytical conditions for resolution of different components were as described earlier [8]. The injector, column and detector temperatures were kept at 250, 230 and 250°C, respectively, with nitrogen as carrier gas at a flow-rate of 12 ml/min. Replicate runs were taken in each case to check the reproducibility.

GC analysis on 5% DEGS stationary phase

In order to obtain better sensitivity for identification of PA, a stationary phase of a lower concentration was used. After initial experiments to determine the retention times of individual amides and acids, the reproducibility of their separation from mixtures and minimum detectable concentrations, urinary extracts of control and DEPA-exposed (head only) rats were analysed for metabolite identification.

A Perkin-Elmer Model 3920 B gas chromatograph (Perkin-Elmer, U.S.A.) was connected to a Chromatopac CR-3A data processor (Shimadzu, Japan) to record the detector response. Analyses were carried out on DEGS stationary phase (5%; 2 m×2 mm I.D. coated on Chromosorb W packed in stainless-steel tubing) at 210°C, with a nitrogen flow-rate of 15 ml/min and with the injector and detector temperatures at 240 and 270°C, respectively. Repeated analyses were carried out to facilitate reliable identification of the metabolites of DEPA.

RESULTS AND DISCUSSION

On the 15% DEGS column, authentic DEPA, EPA and PA from amide mixtures and PhAA and BA from acid mixtures separated well (retention times: 4 min 53 s, 6 min 32 s, 14 min, 5 min 51 s, and 4 min 9 s, respectively). There was no decomposition of the amides or acids tested on stainless-steel columns, as judged from the absence of any spurious peaks when single substances were injected. Under the conditions of analysis up to 100 ng of different compounds could be detected and no effort was made to detect still lower amounts. Typical chromatograms of the standard compounds are shown in Fig. 1.

The urine samples of DEPA-exposed rats (whole body) contained unmetabolized DEPA, EPA and PA in the amide fractions (extracts of step 1), as confirmed by consistent appearance of peaks with retention times comparable with those of reference amides in repeat analyses. A typical chromatogram of a urine extract containing amides is shown in Fig. 2A. In separate experiments it was observed that urine of rats not exposed to DEPA did not contain any of these amides. PhAA and BA were detected in the acid fraction of urine extracts (Fig. 2B).

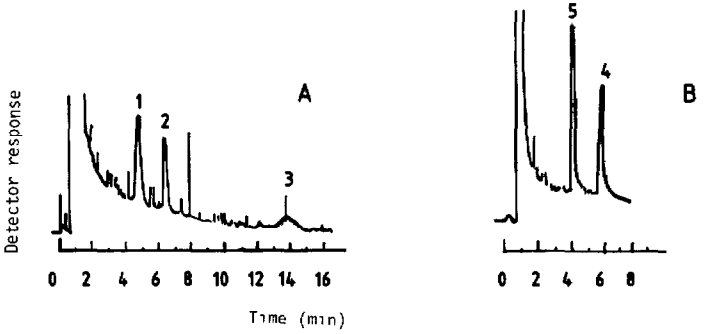

Fig. 1. Resolution of authentic standards (200 ng each) in mixtures of amides (A) and acids (B) on the Nucon gas chromatograph. Column, 15% DEGS; sensitivity, ×100; attenuation, ×16 for amides and ×32 for acids. Peaks: 1 = DEPA; 2 = EPA; 3 = PA; 4 = PhAA; 5 = BA.

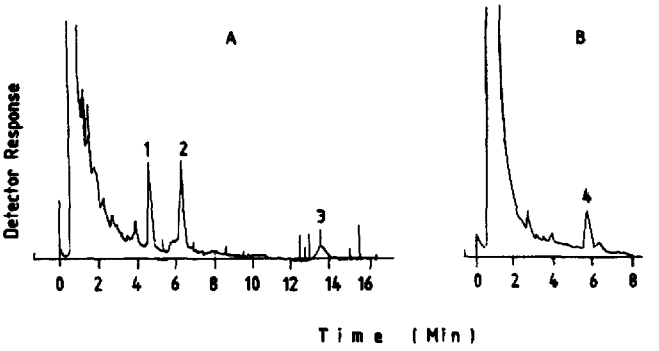

Fig. 2. Chromatograms of urine extracts of rats exposed to DEPA inhalation in whole body exposure, showing amides (A; obtained from the first step) and acids (B; obtained from the second step). Column, 15% DEGS. Settings on Nucon gas chromatograph: sensitivity, ×100; attenuation, ×16. Peaks as in Fig. 1.

On the 5% DEGS column, separation of PA was more satisfactory than on the 15% DEGS column. Under the conditions of analysis its retention time was reduced to 5 min (from 14 min). The retention times of the other compounds of interest, DEPA, EPA and PhAA, were 2.4, 2.7, and 2.5 min, respectively. The retention time of BA, a normal constituent of urine, was 1.9 min under these conditions. At an amplifier setting of ×10, an attenuator setting of ×16 on the gas chromatograph and an attenuator setting of ×4 on the data processor, the minimum detectable amounts of different amides and acids injected in 0.2-μl solutions were: DEPA, 8 ng; EPA, 5 ng; PA, 20 ng; PhAA, 25 ng. The reproducibility of peak areas when aliquots of standard mixtures containing 100 ng were injected four or five times showed a variation of ±6%, and this was considered adequate for the qualitative identification in the present work.

Urine extracts of rats that had inhaled DEPA by head only exposure also showed the presence of the different metabolites (Fig. 3) detected in the experiments with whole body exposure, and the control group contained only BA (Fig. 3).

During sample work-up, total recovery of all the amides of interest was ensured in a different experiment by GC analysis of aliquots from three sequential extractions at pH 7. The last extract showed the absence of DEPA, EPA

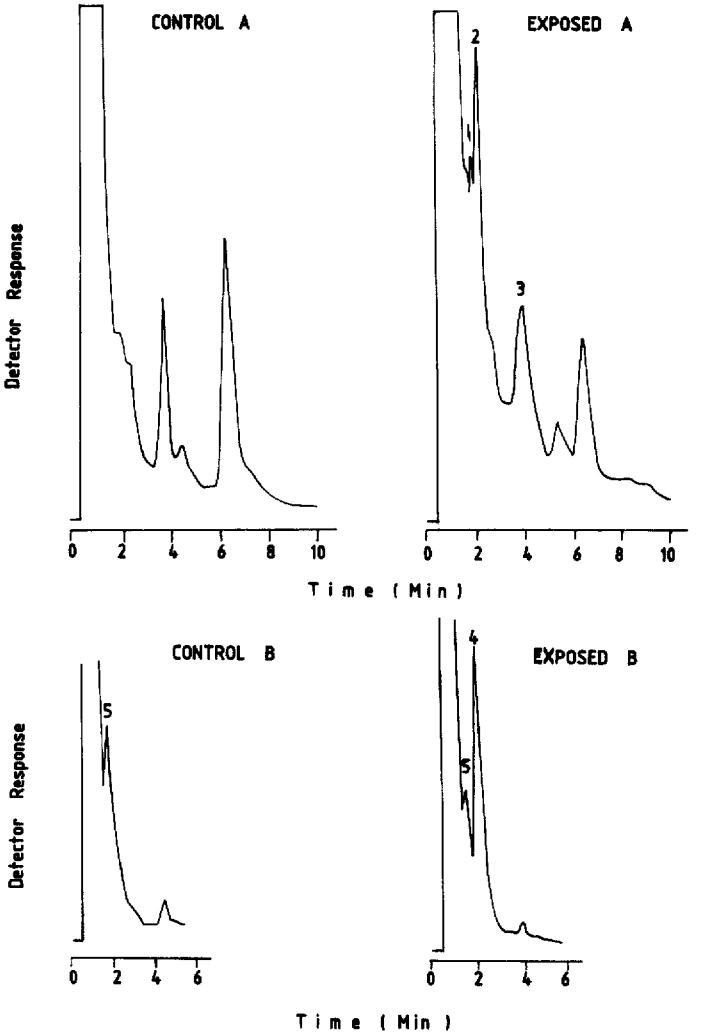

Fig. 3. Chromatograms of urine extracts of control and DEPA-exposed (head only) rats. Chromatograph, Perkin-Elmer Model 3920B; data processor, Shimadzu Chromatopac CR-3A; column, 5% DEGS. GC settings: amplifier, ×10; attenuation, ×16. Data processor attenuation settings: ×4 for A; ×7 for B. Peaks: 1 = DEPA; 2 = EPA; 3 = PA; 4 = PhAA; 5 = BA.

and PA which, if left unrecovered, could give rise to PhAA in a subsequent alkaline hydrolysis step.

N-Dealkylation occurs in biological systems [11]; EPA and PA could arise from progressive N-dealkylation of DEPA. N-Dealkylation of the well known insect repellent N,N-diethyl-*m*-toluamide (DEET) by rat liver microsomes in vitro was reported [12]. Earlier work from our laboratory [8] demonstrated the excretion of EPA in the urine of rabbits when DEPA was applied to the skin at a dose of 50 mg/kg. The GC procedure used for the identification of EPA (and PhAA) employed an OV-17 column. In the present investigation a different chromatographic column (DEGS) was used, and another metabolite, PA, as well as the parent compound, DEPA, were detected in urine. Recently, we have also detected traces of unmetabolized DEPA and PA in urinary extracts of rabbits by this method (S.S. Rao, unpublished results), and hence cannot attribute these metabolites of species-specific or route-dependent metabolism of the compound. Appreciable absorption of DEPA through the skin cannot be ruled out in the rats that had whole body exposure. Such a possibility is minimal in the head only exposure. Since the different metabolites detected were common to both modes of exposure, and also when DEPA was administered to rats by oral or dermal routes (S.S. Rao, unpublished results), it may be assumed that the same metabolic pathway is followed by DEPA in rats whatever the route of entry. Quantitative variation of the metabolites is likely, however, since in vivo extrahepatic drug metabolism can markedly affect the fate of exogenous compounds by generating variable amounts of metabolites identical to or different from those produced in liver [11].

Hydrolytic enzymes with esterase/amidase activities are distributed in animal systems, particularly in liver [13]. Most of the PhAA detected in the urine of DEPA-exposed rats in the present study could be from phenaceturic acid, the glycine conjugate of PhAA, since PhAA is excreted almost entirely as phenaceturic acid [14]. The alkaline hydrolysis step in the sample work-up was intended to release PhAA from such conjugates and make it detectable under the analytical conditions. PhAA can be formed from hydrolysis of PA, EPA and DEPA. A hydrolytic metabolite of DEET, *m*-toluic acid, was earlier found in the urine of rats and rabbits exposed to aerosols of DEET [15].

CONCLUSION

These results demonstrate that DEPA enters systemic circulation when it is inhaled, and crosses air–lung and lung–blood barriers to be biodegraded and excreted. Dealkylation and hydrolysis are operative in the metabolism of DEPA, as seen from the urinary excretion of EPA and PA (from N-deethylation) and PhAA (from hydrolysis).

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