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Note

Determination of 1,3-dinitrobenzene and its metabolites in rat blood by capillary gas chromatography with electron-capture detection

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1,3-Dinitrobenzene (1,3-DNB) is an important chemical intermediate used extensively in the manufacture of aniline dyes, explosives and a number of drugs. It has been shown to be toxic to humans [1–3], rats [4] and rabbits [5], causing methaemoglobinaemia (metHb) in all species and testicular necrosis in the rat [4,6]. In humans 1,3-DNB causes acute metHb, nausea, tachycardia, hypotension and headache. Studies in this research unit have shown this nitro compound to be neurotoxic in conventional male F-344 rats after five daily oral doses of 25 mg/kg, but in germ-free male F-344 rats the signs of neurotoxicity were observed 24 h after a single oral dose of 25 mg/kg [7]. In order to investigate this difference in susceptibility to neurotoxicity a study has been made on the pharmacokinetics of 1,3-DNB in conventional and germ-free rats. This required the development of a sensitive and specific method to determine the parent compound and its metabolites 3-nitroaniline (3-NA) and 3-nitroacetanilide (3-NAce) in small volumes of whole rat blood. The described method is based on the use of capillary gas chromatography (GC) with electron-capture detection (ECD).

EXPERIMENTAL

Materials

1,3-DNB (Sigma, Poole, U.K.), 1,2-dinitrobenzene (1,2-DNB), 3-NA, 4-nitroaniline (4-NA) and 4-nitroacetanilide (4-NAce) (Fluorochem, Glossop, U.K.) were each recrystallized before use and their purity was checked by capillary GC with flame ionization detection (FID). 3-NAce was synthesized by reacting 3-NA (0.5 g) with acetic anhydride (1 ml) containing 1 drop of concentrated sul-

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phuric acid for 2 min at 0°C. Ice cold water was added to the reaction mixture and the solid collected by filtration. The product was recrystallized from ethanol to give a 95% yield (m.p. 154–155°C). The structure of the compound was confirmed by mass spectrometry and its purity checked by GC–FID. Stock standard solutions of the above reference compounds prepared in ethyl acetate at a concentration of 1 mg/ml were stored protected from light at 0°C. Working standards were prepared from these solutions by dilution with ethyl acetate. [^{14}C]1,3-DNB (specific activity 1.91 MBq/m*M*) was a gift from the Central Toxicology Labs., Imperial Chemical Industries (Alderly Park, Macclesfield, U.K.). The purity of the radiolabelled compound was determined by thin-layer chromatography (toluene–methanol–ethyl acetate, 9:1:1).

Ethyl acetate (Fisons, Loughborough, U.K.) of "Distol" pesticide grade and heptafluorobutyric anhydride (HFBA, Pierce, Rockford, IL, U.S.A.) were used without further purification.

Animal studies

Conventional male F-344 rats and germ-free male F-344 rats were dosed orally with 25 mg/kg 1,3-DNB in polyethylene glycol (PEG 600) and serial blood samples were taken from the retro-orbital plexes under light anaesthesia into heparinised tubes at 30 min, 1, 2, 4, 8, 12, 18 and 24 h after dosage. Samples were stored at −20°C until required for analysis.

Extraction and derivatization

All extractions were carried out in Eppendorf disposable microcentrifuge tubes. A 50-μl sample of rat blood was spiked with the internal standards 1,2-DNB (50 or 5 ng), 4-NAce (50 or 5 ng) and 4-NA (50 or 5 ng), and 100 μl ethyl acetate was added. After vortex-mixing and centrifugation (2 min at 5600 *g*) the ethyl acetate layer was transferred into a silanized 1-ml Reacti-vial and 1-μl aliquots were taken for the GC–ECD analysis of 1,3-DNB and 3-NAce. A 50-μl aliquot of the solvent extract was then reacted with 10 μl HFBA for 20 min at room temperature in a separate silanized 1-ml Reacti-vial. The reaction mixture was taken to dryness at room temperature under nitrogen and then redissolved in ethyl acetate for GC–ECD analysis of 3-NA.

Gas chromatography

The analyses were performed using a Carlo Erba Fractovap 4160 gas chromatograph equipped with a HT-25 ^{63}Ni (10 mCi) electron-capture detector operated in the constant-current mode (voltage 50 V, pulse width 1 μs). The GC separations were made on a 20 m × 0.32 mm fused-silica capillary deactivated according to Grob and Grob [8] and statistically coated with OV-1701 phase (film thickness 0.25 μm). At the inlet end of the column a 1 m length of deactivated uncoated fused-silica capillary was attached to the analytical column by means of a glass press-fit connector [9,10]. Samples were injected by split injection (10:1 split ratio). The carrier gas was helium at a column flow-rate (measured at 200°C) of 5 ml/min and the detector make up gas was nitrogen at a flow-rate of 30 ml/min. For the analysis of 1,3-DNB and 3-NAce the column was initially held at 100°C

for 1 min, then its temperature was increased at 20°C to a final temperature of 250°C. After derivatization 3-NA was determined either using the same oven temperature conditions or with the oven maintained at 210°C. Gas chromatograms were recorded with a Shimadzu C-R3A Chromatopac computing integrator.

Calibration curves

Quantitation of 1,3-DNB, 3-NAce and 3-NA was made by reference to standard calibration curves constructed with each batch of samples analyzed. The standard curves were prepared by analysing 50-μl aliquots of blank rat blood containing 20–200 ng 1,3-DNB, 3-NAce and 3-NA and 50 ng of each of the internal standards. When the concentration levels were expected to be less than 20 ng per 50-μl sample, one tenth of the amounts of 1,3-DNB, 3-NAce, 3-NA and internal standards were used for the calibration curves. Peak-height ratios analyte/internal standard against nanograms of analyte added were plotted for 1,3-DNB and 3-NA and peak-area ratios for 3-NAce.

Gas chromatography–mass spectrometry

The identification of blood metabolites was carried out using a VG 70-SEQ mass spectrometer interfaced to a Hewlett-Packard Model 5700 gas chromatograph. The 25 m$\times$0.32 mm SE-54 fused-silica capillary, which was inserted directly into the ion source, was operated with an oven temperature held at 80°C for 1 min, then programmed at 30°C/min to 280°C. Samples were introduced by on-column injection. The mass spectrometer was used in negative-ion chemical ionization mode with ammonia as reagent gas. The source temperature was 200°C, the electron energy 50 eV and the emission current 500 μA.

RESULTS AND DISCUSSION

1,3-DNB and its metabolites 3-NAce and 3-NA were initially identified by capillary GC with mass spectrometry in ethyl acetate extracts from the blood of rats dosed with 50 mg/kg 1,3-DNB. The parent compound and 3-NAce could be detected with high sensitivity using the electron-capture detector. However, in order to achieve a good detector response for 3-NA this metabolite had to be first derivatized with an electrophoric derivatizing reagent. HFBA was selected for this purpose since the resulting fluoroacyl derivative (3-NA-HFB) was readily formed in quantitative yield, was volatile with good GC properties and provided a six-fold increase in sensitivity to ECD. Fig. 1 shows the GC separation and relative detector response of the standard compounds before and after derivatization with HFBA.

Ethyl acetate was an effective solvent for extracting all three nitro compounds and their respective internal standards from blood. A single extraction with two volumes of ethyl acetate recovered 92% of the radioactivity from the blood of rats collected 2 h after dosage with [^{14}C]1,3-DNB. For the analysis of the parent compound and 3-NAce in rat blood no concentration or purification of the ethyl acetate extract was necessary. Attempts to quantitate all three compounds simultaneously after the derivatization step resulted in a loss of 1,3-DNB during

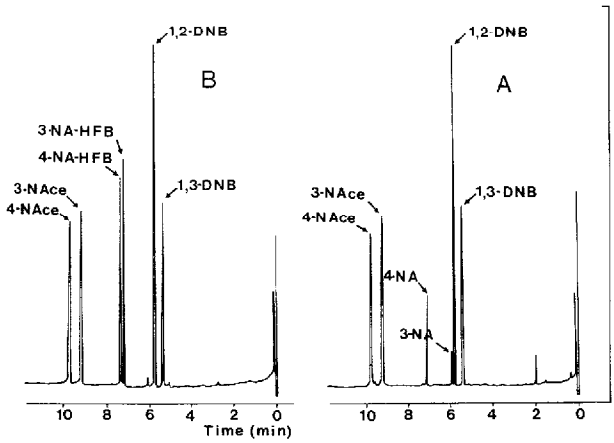

Fig. 1. Chromatogram of (A) an authentic mixture containing 1 ng/μl each of 1,3-DNB, 1,2-DNB, 3-NA, 4-NA, 3-NAce and 4-NAce and (B) an authentic mixture containing 1 ng/μl each of 1,3-DNB, 1,2-DNB, 4-NAce and 3-NAce and 250 pg/μl each of the heptafluorobutyryl derivatives of 3-NA (3-NA-HFB) and 4-NA (4-NA-HFB). For GC–ECD conditions see text.

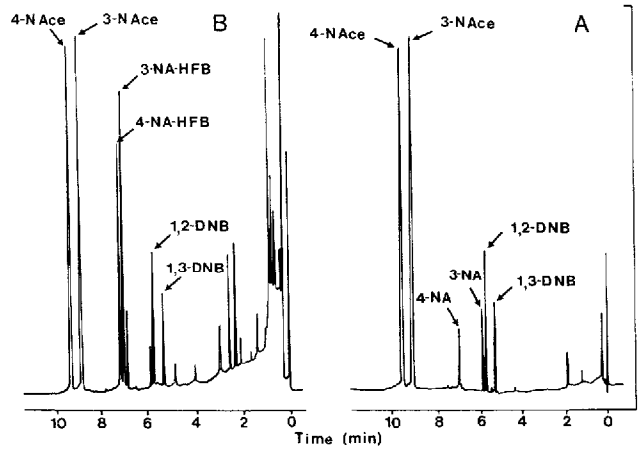

Fig. 2. Chromatogram of (A) the analysis of 1,3-DNB and 3-NAce in an extract of blood from a rat 3 h after dosage with 25 mg/kg 1,3-DNB and (B) the analysis of 3-NA as its heptafluorobutyryl (HFB) derivative in the same blood extract. For GC–ECD conditions see text.

the evaporation of the extract to remove excess HFBA. Fig. 2A shows a gas chromatogram from the determination of 1,3-DNB and 3-NAce in a rat blood sample taken 3 h after dosage with 25 mg/kg 1,3-DNB and Fig. 2B shows a chromatogram from the determination of 3-NA as its heptafluorobutyryl derivative in an extract from the same blood sample. The analysis of blank rat blood extracts gave few background peaks, none of which interfered with the measurement of 1,3-DNB, its two metabolites or the three added internal standards. Standard curves used for quantitation exhibited good linearity over the concentration ranges measured. The accuracy of the method was determined from recovery experiments of authentic standards added to blank rat blood samples at concentrations of 6 and 2

TABLE I

RECOVERY OF 1,3-DNB AND ITS TWO METABOLITES IN SPIKED BLOOD SAMPLES ($n=6$)

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Recovery (mean ± S.D.) (%)
1,3-DNB	2.0	2.22 ± 0.074	111.0 ± 3.70
	6.0	6.61 ± 0.204	110.3 ± 3.40
3-NAce	1.0	1.02 ± 0.011	101.8 ± 1.11
	3.0	2.94 ± 0.062	98.14 ± 1.86
3-NA	0.25	0.26 ± 0.037	104.4 ± 14.9
	1.5	1.55 ± 0.153	103.6 ± 10.2

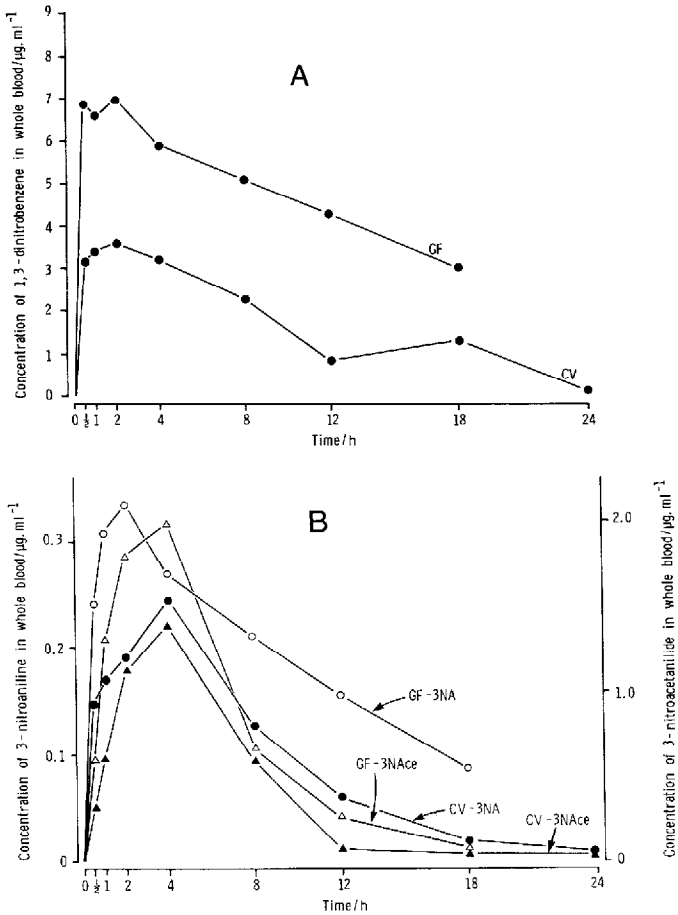

Fig. 3. Blood concentration–time curves of 1,3-DNB (A) and 3-NA and 3-NAce (B) in conventional (CV) and germ-free (GF) rats after oral dosage of 25 mg/kg 1,3-DNB. Each point is a mean determination from four rats.

μg/ml for 1,3-DNB, 3 and 1 μg/ml for 3-NAce and 1.5 and 0.25 μg/ml for 3-NA. The precision of the method was determined from replicate analyses at these same concentrations. The results from these analyses are presented in Table I. The absolute recoveries of the three compounds using 1,2-DNB, 4-NAce and 4-NA as external standards gave a recovery (mean ± S.D.) for 1,3-DNB of 99.9 ± 4.1%, for 3-NAce of 92.9 ± 6.7% and for 3-NA of 103.8 ± 5.8%. The sensitivity of the method for 1,3-DNB and its two metabolites can, if required, be improved by injecting the extract into the capillary column using either the splitless or on-column injection technique. The lower limit of detection is in the order of 10 ng/ml of blood.

The application of the method to a study of the blood concentration–time profiles in conventional and germ-free rats following a 25 mg/kg oral dose of 1,3-DNB is shown in Fig. 3. A more detailed study of the pharmacokinetics and rate of elimination of 1,3-DNB in the rat will be the subject of a separate publication.

In conclusion the described method provides an accurate and precise method for measuring 1,3-DNB and its two major metabolites in blood. The ability to perform the analysis on small sample volumes with high sensitivity makes the assay very suitable for pharmacokinetic studies in the rat.

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