ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION OF 2,3-DIMETHYL-2,3-DINITROBUTANE.

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ABSTRACT

The compound 2,3-dimethyl-2,3-dinitrobutane (DMNB) is routinely used to tag explosives to enable detection during covert transport for terrorist purposes. The present study of the absorption, metabolism, distribution and excretion of DMNB in rats was carried out to determine the pharmacokinetics after percutaneous exposure and whether any toxic metabolites were formed. DMNB was absorbed slowly into the body over a 6 hour application period and formed a depot which continued release into the blood for some time after dosing. A proportion of the applied dose was retained within the blood and tissues 168 hours after application, indicating a prolonged residency within the body. The DMNB was excreted in the urine, expired air and faeces. Whole body autoradiography showed that the DMNB distribution to fatty and glandular tissues. There was evidence of metabolism, 5-6 possible metabolites being excreted in the urine, all of which appeared to be volatile, excluding the possibility of conjugation with glucuronic acid, sulphate or glutathione.

INTRODUCTION

The need to detect explosives being used or transported for terrorist purposes has led to the inclusion of a “taggant” at the point of manufacture, which can be detected by analysis of the air in the vicinity of the explosive. The compound 2,3-dimethyl-2,3-dinitrobutane (DMNB) is now in use as a taggant in plastic explosive because it is not used for other purposes and has a suitable volatility. Prior to its adoption as a taggant, DMNB was the subject of toxicity studies commissioned by the US Government (Weeks, 1993), to ensure that it was safe to use in this way.

The US study reported toxicity tests of acute, sub-chronic and developmental toxicity in rodents. Mutagenicity and carcinogenicity testing was undertaken *in vitro* along with a number of tests in insects, birds and fish. Acute toxicity was measured after oral, dermal, intraperitoneal and inhalation exposure, and the sub-chronic toxicity test was a 90-day feeding trial. These studies were reviewed by Shayer (2002) who concluded that there was no evidence that DMNB causes skin or eye irritation, that it has low vapour toxicity, is non-mutagenic, non-carcinogenic and has little or no developmental toxicity. There is evidence of toxicity by the oral route and after inhalation of the dust. The "metabolism" study reported by Weeks (1993) showed that DMNB is retained within the bodies of rats for a long period (greater than 3 days) and highlighted two gaps in our knowledge of its toxicology. Whether the chemical could be absorbed thorough the skin in significant quantities and the identity of any metabolites formed.

The objectives of the study reported here were to further define the pharmacokinetics of DMNB in the rat and identify any metabolites formed.

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METHODS

[^{14}C]-labeled DMNB was synthesized by Biodynamics Ltd and was of >95% radiochemical purity. The unlabelled DMNB was supplied by Royal Ordinance (Bridgwater, UK) and was commercial grade (>98%, NMR).

Preparation and dosing of animals

Animals were close clipped 245 hours before dosing and dose sites were free of any overt damage when the dose was applied. [^{14}C]DMNB was applied to 3 cm² (~10% of the body surface area) of the back at a nominal dose of 36 mg.kg^{-1} (40 µg.kg^{-1}). The dosing site was covered with a semi-occlusive "jacket" after dosing and the animals housed individually in metabolism cages for the remainder of the study. After 6 hours the jackets were removed, the dosing site washed and the animals returned to the metabolism cages.

Pharmacokinetics

Twelve male and twelve female rats received a single topical dose of [^{14}C]-DMNB at a nominal dose level of 36 mg/kg. Blood samples (for plasma preparation) were then obtained either from the tail vein (ca. 1 ml) or as a terminal sample (ca. 8 ml) at the following times post dose administration:

No. of animals	Collected from the tail vein (ca. 1 ml)		Terminal sample (ca. 8 ml)
3 male, 3 female	Pre dose	4 hour	6 hour
3 male, 3 female	-	8 hour	24 hour
3 male, 3 female	48 hour	72 hour	96 hour
3 male, 3 female	120 hour	144 hour	168 hour

For terminal sample collection, animals were anaesthetised with Isofluorane and a terminal blood sample taken by cardiac puncture prior to being killed by cervical dislocation. Whole blood samples were immediately centrifuged (ca. 1500 x g for 15 minutes), plasma was harvested for quantitative radiochemical analysis. The remaining red blood cells were discarded.

Excretion/balance studies

Three male and three female rats received a single topical dose of [^{14}C]-DMNB at a nominal dose level of 36 mg/kg. Urine (collected in a container cooled in solid carbon dioxide), faeces and expired air were collected separately at the following intervals:

Urine:	0 – 6, 6 – 24 and at 24 hour intervals up to 168 hours
Faeces:	0 – 24 and at 24 hour intervals up to 168 hours
Expired air:	0 – 6, 6 – 24 and at 24 hour intervals up to 168 hours

At 168 hours after dose administration the rats were killed by cervical dislocation. The application site skin removed for measurement of radioactive content.

Urine was collected into pre-weighed containers surrounded by solid CO_2 and the weight of each sample recorded. Duplicate aliquots were taken for LSC and the sample retained at ca. -20°C. Faeces were collected into individual pre-weighed containers and the weight of each sample recorded. Prior to analysis faeces samples were homogenised into a paste with water. Duplicate aliquots were taken for

combustion analysis/LSC and the sample retained at ca. -20°C. Carcasses and Skin (Dose sites) were retained at ca. -20°C and digested in a basic solution prior to analysis.

During a preliminary study, duplicate aliquots of skin samples were taken for combustion analysis/LSC and the sample retained at ca. -20°C. These tissues were collected into individual pre-weighed containers and the weight of each sample recorded. Prior to analysis samples were thoroughly scissor minced. Duplicate aliquots were taken for combustion analysis/LSC and the sample retained at ca. -20°C.

The cotton wool and absorbent paper used to wash the dosing sites were extracted three times with acetone. Duplicate aliquots of each extract were taken for LSC. Expired carbon dioxide was trapped using ethanol:water (50:50 v/v) in trap 1 and ethanolamine: 2-ethoxyethanol (1:3 v/v) in traps 2 and 3 connected in series. Trapping solutions were transferred into pre-weighed collection vessels and the weight of each sample recorded. Duplicate aliquots of each wash were taken for LSC.

The radioactivity in formulations, urine, plasma, site washing extracts and expired air trap samples was determined directly by liquid scintillation counting in Ultima Gold scintillant (Packard scintillation counter). Aliquots of faecal homogenates and tissue samples were combusted (Packard Sample Oxidiser 307) and the $^{14}CO_2$ produced trapped with the carbon dioxide absorbent Carbosorb E^+, which was mixed with the scintillant Permafluor E^+ prior to counting.

Carcasses and skin were dissolved at 55°C in a mixture of sodium hydroxide:methanol:Triton X-405:water (80/300/100/600 w/v/v/v) prior to estimation of radioactivity content by LSC.

The plasma DMNB concentration versus time curves obtained following topical administration were analysed using PCModfit (Version 3.0). The kinetic data was characterised by a non-compartmental analysis (NCA).

HPLC Analysis

Dose formulation, urine samples and plasma samples were analysed by direct injection onto the HPLC column. Due to the high volatility of DMNB it was not possible to extract plasma or faeces. Faeces samples were not analysed by HPLC. Plasma samples were centrifuged following addition of trifluoroacetic acid. Two HPLC methods were used. Method 1 separated samples on a Lichrospher 5µ RP18-endcapped 250 x 4.6 mm from a mobile phase Acetonitrile:Water (30:70, isocratic) at a flow rate of 1.5 $mL.min^{-1}$ at ambient temperature with UV detection (210nm). This system was used for radiochemical purity checks and initial profiling work performed on urine, air traps and site washings. Method 2 was developed for further urine, plasma and site washings analysis and was the same as method 1 with an acetonitrile:0.5%formic acid gradient (10:90 to 90:10 over 45 minutes).

Quantitative Whole-body Autoradiography study (Group 8)

Six male and six female rats received a single topical dose of [^{14}C]-DMNB and 1 male and 1 female were killed by overdose of CO_2 at each of 6, 24, 72, 96, 168 and 240 hours after dosing. Animals were immediately frozen in a hexane/solid CO_2 mixture and retained at ca. -20°C. The cadavers were sectioned on a Leica CM3600 cryomicrotome and the sections subjected to whole-body autoradiography using procedures based on the work of Ullberg (1954). Three sections at up to five different levels of the rat body to include as many tissues as possible.

Level A: exorbital lachrymal gland

Level B: intra-orbital lachrymal gland
Level C: Harderian gland/adrenal gland
Level D: thyroid
Level E: brain and spinal cord

Due to the highly volatile nature of DMNB, autoradiography was carried out on fresh frozen sections. The plates were placed in light-tight cassettes and stored in a freezer at ca. -80°C for seven days. At the end of the exposure period, plates were scanned using a Fuji BAS 1500 scanner. Distribution of radioactivity was determined and quantified using the Fuji BAS 1500 bio-image analyser and associated Tina and SeeScan software. Tissue concentrations of radioactivity were determined using calibrated autoradiographic microscales produced by Amersham International enabling the expression of tissue radioactivity content in μg equiv./g.

RESULTS

Pharmacokinetics and blood levels

In male and female rats treated cutaneously with 36 mg/kg ^{14}C-DMNB (site occluded) for six hours, the radioactivity was slowly absorbed into the blood reaching a maximum concentration 24 hours after application which was higher in female than male animals (figure 1). The concentrations of radioactivity then reduced with apparent terminal half-lives of 63.23 hours (female) and 40.43 hours (male), but some radioactivity remained in the blood at 168 hours after dosing. Analysis of the blood samples by HPLC using a radiometric detector showed that the radioactivity in the blood was associated with only one compound, which was chromatographically identical to DMNB. The changes in the blood concentrations indicate that the rate of elimination from the blood is lower at later time points than earlier.

Excretion/Balance

Radioactivity was excreted in the urine, faeces and exhaled on the breath as illustrated in Figure 2. Over the 168 hours of the experiment male animals excreted a total of 46 ± 3.5% applied dose, and female animals 38 ± 8%, in urine, faeces and expired air. At the end of the 168 hours the total radioactivity recovered from all sources, including that remaining in the carcass, was measured (figure 3). The majority of the excreted radioactivity was excreted in the urine and expired air with a small percentage in the faeces. Only 2.28 ± 0.6 % (female) or 0.6 ± 0.4% (male) of the applied dose remained in the carcass at 168 hours after exposure. Recoveries were 70.39 % in females and 64.42% in males.

A preliminary experiment where DMNB was placed in metabolism cages in a petri dish showed that there was sufficient material evaporated in the 168 hours to be detected in the CO_2 traps of the apparatus.

Distribution

Whole body auto-radiographs of animals killed between 6 and 240 hours after percutaneous application showed distribution of radioactivity into fatty and glandular tissues of the body (illustrated in figure 4). Whole body autoradiography was carried out on fresh frozen sections as freeze-drying resulted in loss of almost all the radioactivity.

Semi-quantitative scanning densitometry showed that the radioactivity reached the highest concentrations in the preputial gland of the male and clitoral gland of the female, the nasal gland and mucosa, white fat, liver and kidney. Significant concentrations of radioactivity were also found in the lung, lachrymal glands and the harderian gland. As in the blood the highest concentrations were observed

at 24 hours after dosing and decreased thereafter, though some radioactivity remained in the kidney, fat, nasal gland and mucosa, and preputial or clitoral gland 240 hours after dosing.

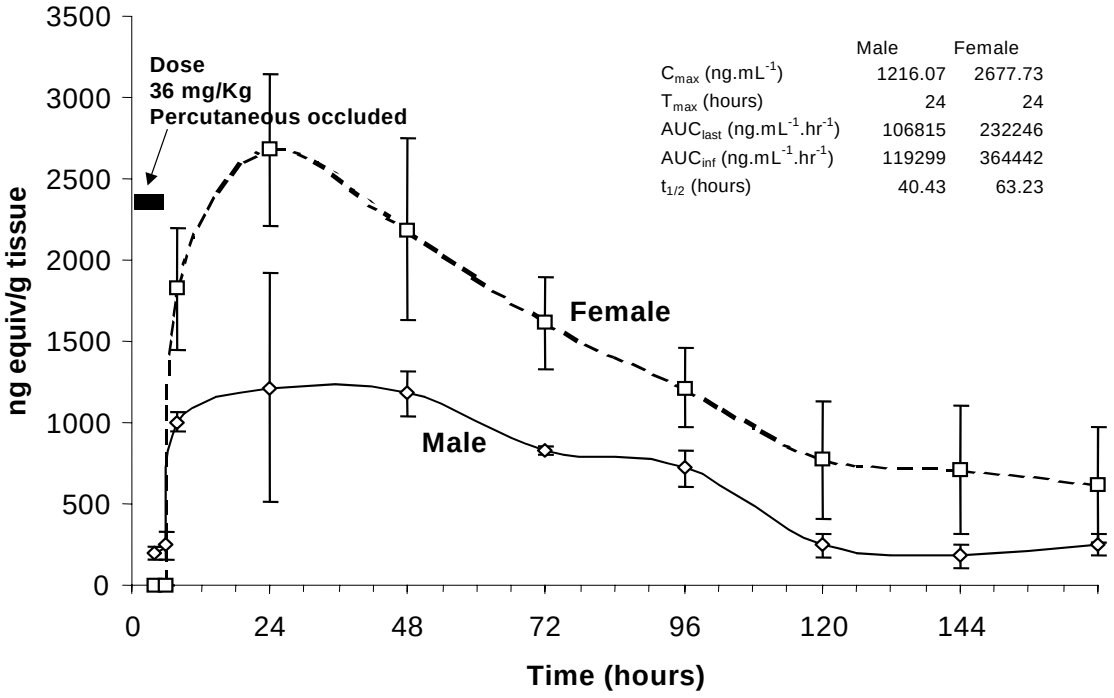

Figure 1: Plasma concentrations in male and female rats after percutaneous exposure to 36 mg.kg^{-1} for 6 hours (indicated by black bar). Pharmacokinetic parameters calculated from non-compartmental analysis of the curves fitted in figure 1 Annex C are inset. Points are mean ± SD of three individuals at each sample time.

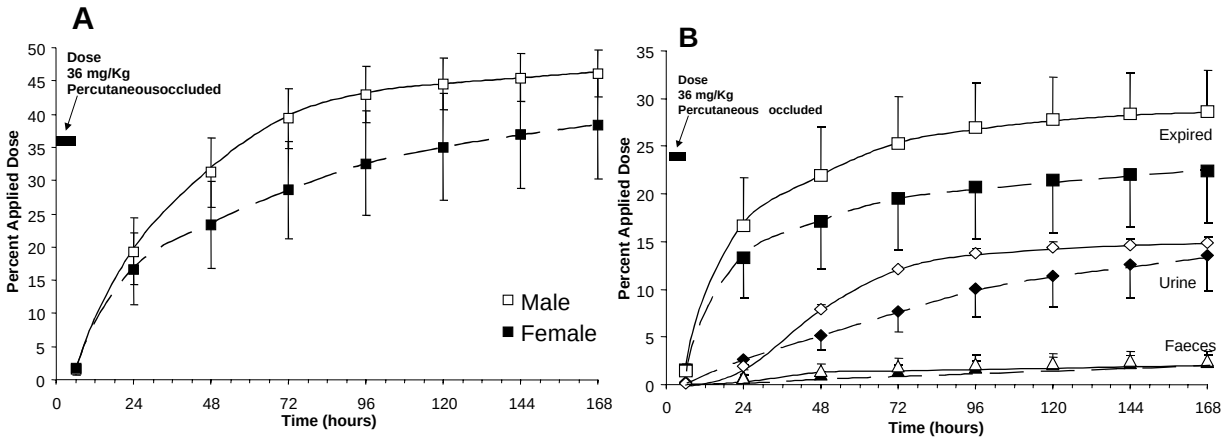

Figure 2: Radioactivity excreted by male (open symbols) and female (closed symbols) rats for 168 hours after a 6 hour percutaneous application of 36 mg.kg^{-1} ^{14}C-DMNB (indicated by black bar). Results are expressed as total percentage of applied dose excreted and points are mean ± SD (n=3). A – total excretion by all routes, B – excretion in expired air (squares), urine (diamonds) and faeces (triangles).

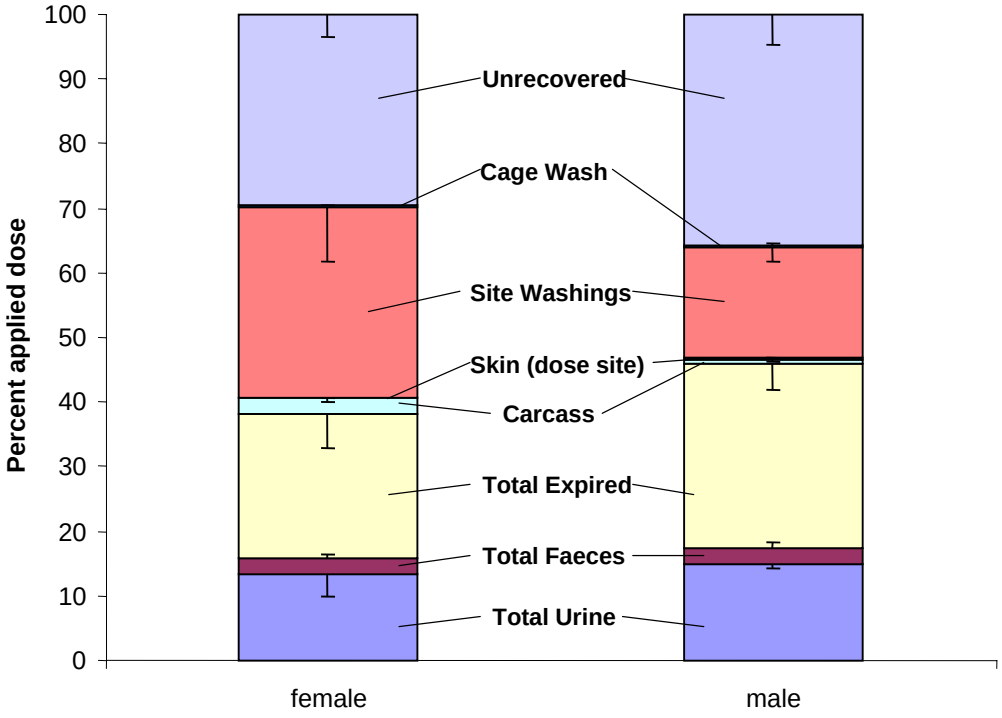

Figure 3: Total radioactivity recovered from male and female rats 168 hours after a 6 hour percutaneous exposure to 36 mg.kg^{-1} DMNB. Bars and mean ± SD (n=3).

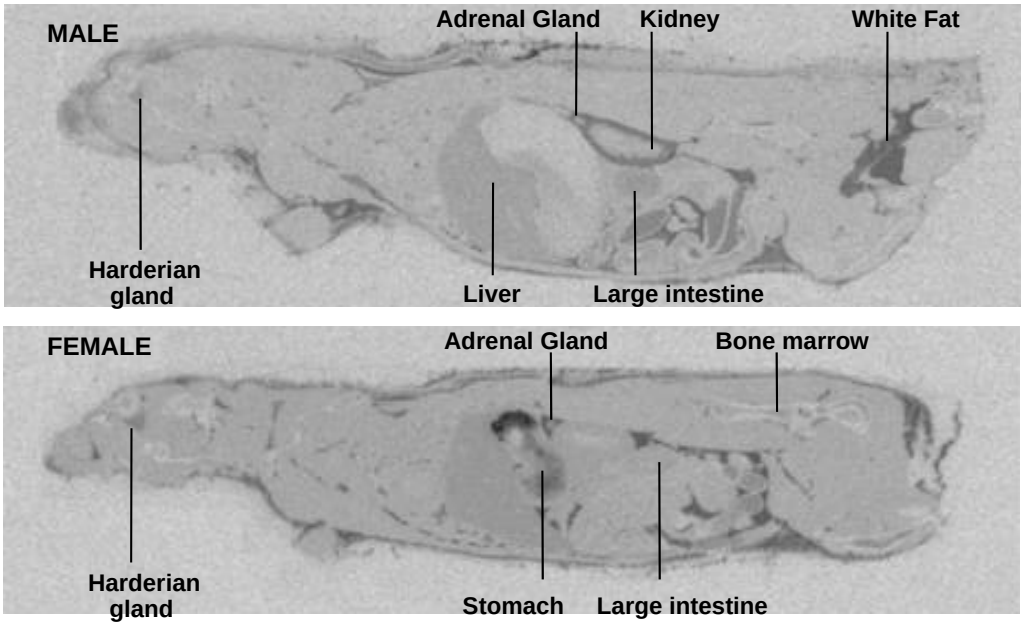

Figure 4: Illustrative whole body auto-radiographs of rats treated percutaneously with ^{14}C-DMNB (24hrs, Level C- Harderian gland/adrenal gland). Dark areas indicate radioactive material present in the tissues, the darkest areas have the most radioactivity, lighter areas less.

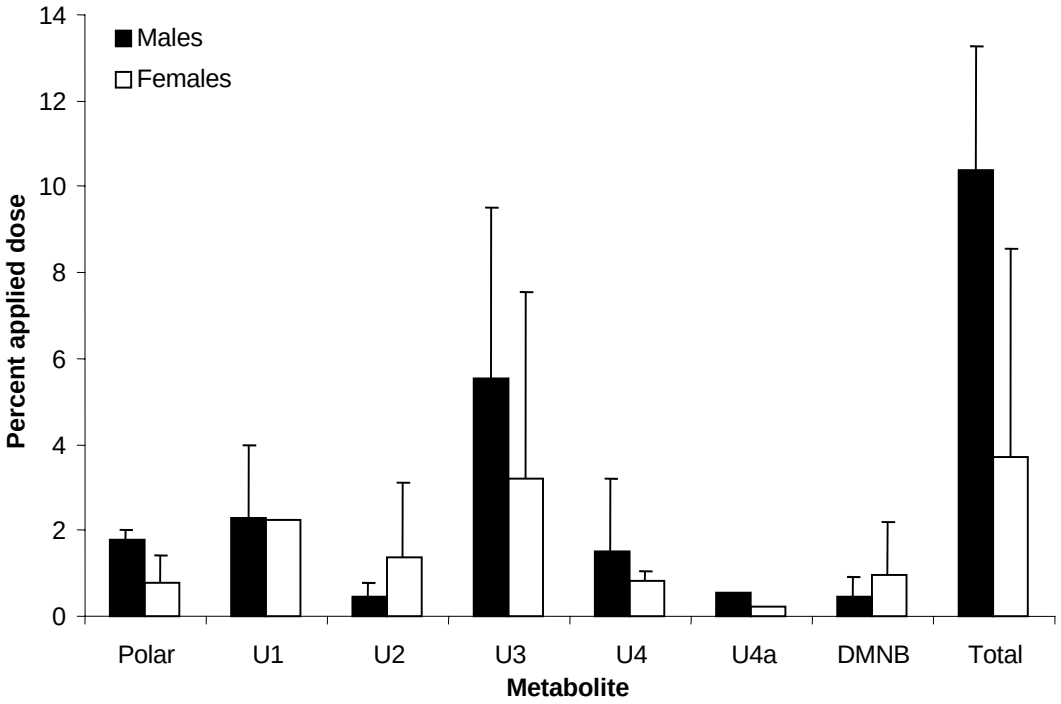

Figure 5: Profile of urinary metabolites analysed by HPLC excreted over 168 hours following a percutaneous dose of DMNB (36 mg.kg^{-1}). Polar metabolites elute in the solvent front, the remaining metabolites were separated and have been assigned a code number.

Metabolite Profiling

The loss of almost all the radioactivity in the whole body autoradiograph sections after freeze drying indicates that the radio-labelled material was volatile. The volatility of the material made extraction and analysis by HPLC of the faeces and the contents of the traps for expired material very difficult, and no metabolites could be identified from these sources.

Analysis of the urine however, showed the presence of four distinct metabolites, labelled U1, U2, U3 and U4, with an indication of some polar metabolites eluting in the solvent front and a possible fifth metabolite associated with U4 and labelled U4a (figure 5). The urinary metabolites accounted for between 4.7-12.7% of the applied dose. The profiles of urinary metabolites were qualitatively similar in males and females. Very little radioactivity remained in the urine after lyophilisation, indicating that there were few, if any, conjugates formed with glucuronic acid, sulphate or glutathione.

There was insufficient material excreted in the faeces, and extraction of the contents of the air traps was not suitably efficient to enable meaningful analysis.

DISCUSSION

This study has more completely defined the pharmacokinetics and distribution of DMNB in the rat and given some indication of the extent of metabolism.

Pharmacokinetics

The increase in the concentration of radioactivity in the blood to reach a maximum at 24 hours is consistent with either a slowly absorbed material forming a depot within the skin which slowly released material, or the formation of a depot within the body after absorption. The observation that absorption rates in the rats are greater than those in man is inconsistent with the DMNB behaving as a slow penetrant, and the partitioning of material into fatty tissue, see below, supports the conclusion that the "depot" is formed in the fatty tissue of the body after absorption. The plasma half-lives of between 40 and 60 hours are high and indicate that the DMNB will be retained within the body for a long time after absorption. Moreover, the rate of elimination of DMNB from the plasma at later times (120-168 hours) appears to be lower than at earlier times, also indicating that the material could be retained in the body for prolonged periods of time. This confirms the observations of Weeks et al (1993) that the "metabolism" of DMNB after intraperitoneal and oral dosing was not "a simple first order event". It is tempting to specutlate tht the initial elimination is driven by the concentration in the blood whereas at later times the rate of release from the depot becomes rate limiting.

Given the apparent prolonged retention of DMNB in the body after a single percutaneous dose, the possibility of accumulation after multiple and/or prolonged exposures cannot be excluded. A multi-compartment pharmacokinetic analysis of the existing data and the development of a physiologically based pharmacokinetic (PBPK) model to explore the possible effects of multiple dosing should be considered.

Absorption and distribution.

The skin of each animal was exposed to DMNB for 6 hours, and then swabbed clean with acetone. After 48 hours, only a small percentage of the applied dose was found in the skin. This is inconsistent with the apparent persistence of DMNB after this time being due only to continued delivery from the application site. This result indicates that, in addition to continued delivery from what remains in the skin, DMNB is partitioning back out of the fat and muscle (where the largest bio-accumulation was located), into the systemic circulation from whence it is excreted.

The tissue distribution is demonstrated by the results of the whole body auto-radiographic study. It is clear that the DMNB partitions preferentially into fatty tissue, white fat and glands that produce lipid rich secretions such as the nasal gland, nasal epithelia and the preputial or clitoral gland. The DMNB in the fatty tissues probably forms the "depot" which continues to deliver compound to the blood long after it has been absorbed from the skin.

Excretion

The considerable percentage of the applied dose found in the exhaled air could be unchanged DMNB, the products of metabolism, or CO_2 as the final product of oxidative metabolism. The excretion of the remaining absorbed dose primarily in the urine as opposed to the faeces is also consistent with a volatile small molecule.

Metabolism

The results of this study clearly indicate that there is a degree of metabolic conversion of DMNB carried out in the body, although the identities of the metabolites have not been established. There are a number of metabolic conversions to which DMNB might be subjected. Some of these conversions can be discounted as occurring in the rat from the results of the current study. Possible metabolic transformations are:

1. Conjugation of the nitro group with glucuronic acid, sulphate or glutathione.
2. Denitration.
3. Oxidative demethylation
4. Hydroxylation of an aliphatic carbon.
5. Reduction of the nitro group to an amino group

Conjugation can be discounted since its products are non-volatile, would have been detected in urine, and furthermore no evidence of such metabolites was found (the radioactivity in the lyophilised urine is consistent with residual volatile metabolites: 1.4%). Denitration and oxidative demethylation are known to occur, but are very rare transformations. The most likely routes of metabolism for DMNB are reduction of the nitro groups to an amine, via the hydroxylamine, and hydroxylation of the aliphatic chain to form an alcohol.

Though none of the metabolites of DMNB have been identified in this study it is very clear that they are volatile chemicals which are not conjugated.

CONCLUSIONS

This study has demonstrated that DMNB is absorbed through the skin and retained within the body for a prolonged period after absorption. The distribution of DMNB indicates that it is preferentially partitioned into fatty tissues and is excreted primarily in the urine and on the expired air. DMNB is metabolised to at least 5 metabolites excreted in the urine, though these have not been identified there behaviour during analysis indicates that they are volatile.

The absence of any radioactivity form lyophilised urine indicates that DMNB or any of its metabolites is not appreciably conjugated with any of the usual molecules such as glucuronic acid, sulphate or glutathione.

Though the general toxicology of DMNB give no indication that the molecule is likely to be toxic to humans, its prolonged residence time in the body indicates that the identity of its metabolites should be determined to eliminate the formation of any reactive molecules.

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