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DIMETHYLMORPHOLINOPHOSPHORAMIDATE (DMMPA) PERSISTENCE IN VARIOU--ETC(U)
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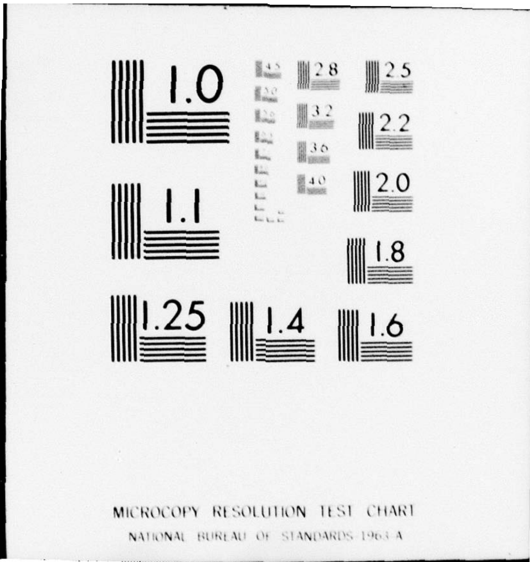
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REPORT

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DIMETHYLMORPHOLINOPHOSPHORAMIDATE (DMMPA) PERSISTENCE IN VARIOUS TISSUES OF THE RAT

R.M. Dawson, B.R. Lakeland, M. Poretski, and M.P. Bladen

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(6) DIMETHYLMORPHOLINOPHOSPHORAMIDATE (DMMPA) PERSISTENCE IN VARIOUS TISSUES OF THE RAT

(10) R.M. Dawson, B.R. Lakeland, M. Poretski and M.P. Bladen

ABSTRACT

The persistence of dimethylmorpholinophosphoramidate (DMMPA) in various tissues of the rat was examined in order to assess whether it could pose a threat to chronically exposed animals. The material was purified by filtration through alumina in ether followed by fractional distillation. Significant amounts of DMMPA were found in the liver, kidneys and brain of rats 4 hours after i.p. injection, but only traces were recovered at 48 hours after injection. Recovery of DMMPA in the urine over 48 hours was approximately 60%, and almost all of this was excreted in the first 24 hours. DMMPA had no effect on a rat phrenic nerve-diaphragm preparation, nor on the guinea pig right atrium. DMMPA is unlikely to present a chronic threat to the exposed animal or human.

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16. ABSTRACT (if this is security classified, the announcement of this report will be similarly classified):

The persistence of dimethylmorpholinophosphoramidate (DMMPA) in various tissues of the rat was examined in order to assess whether it could pose a threat to chronically exposed animals. The material was purified by filtration through alumina in ether followed by fractional distillation. Significant amounts of DMMPA were found in the liver, kidneys and brain of rats 4 hours after i.p. injection, but only traces were recovered at 48 hours after injection. Recovery of DMMPA in the urine over 48 hours was approximately 60%, and almost all of this was excreted in the first 24 hours. DMMPA had no effect on a rat phrenic nerve-diaphragm preparation, nor on the guinea pig right atrium. DMMPA is unlikely to present a chronic threat to the exposed animal or human.

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DIMETHYLMORPHOLINOPHOSPHORAMIDATE (DMMPA)

PERSISTENCE IN VARIOUS TISSUES OF THE RAT

INTRODUCTION

Dimethylmorpholinophosphoramidate (DMMPA) is a non-toxic liquid which resembles certain nerve agents in its physical properties and its ability to be absorbed through the skin. It is a suitable total intake simulant for studying the effectiveness of chemical defence procedures (1). As a prelude to the limited use of DMMPA on humans a substantial amount of data on the acute and chronic toxicity of DMMPA was gathered by Coleman (2,3). This project did not include studies of the persistence of DMMPA in animal tissues, nor its effect on the neuromuscular junction. Such studies are the subject of this paper. The analysis, purification and stability of DMMPA were also examined.

MATERIALS AND METHODS

Technical DMMPA was provided by Terochem Laboratories Ltd., Edmonton, Canada. Another sample of DMMPA was obtained in good yield by synthesis according to the procedure of Hansen *et al* (4). Its identity was confirmed by comparison with an authentic, pure sample provided by Defence Research Establishment, Suffield, Canada. Thin-layer and gas chromatography were the means of comparison. In addition, the n.m.r. spectrum of the synthesised sample in $CDCl_3$ was recorded on a Varian instrument operating at 60MHz. The spectrum was identical with a published spectrum of DMMPA (5).

Assays

Total phosphorus was determined by King's method (6).

Inorganic phosphorus was determined by the Fiske-Subbarow method (6).

DMMPA was determined by a modification of the method of McNally and Adie (1). Chloroform (14.0 ml) and 1.0 ml 1M NaOH were added to 10 ml aqueous solution of DMMPA and the mixture was shaken 2 x 30 s. The chloroform phase was dried with anhydrous sodium sulphate and concentrated

to 3-5 ml on a rotary evaporator at room temperature. It was then transferred to a 10 ml test tube, evaporated to dryness, and 0.5-5.0 ml n-hexane added. The hexane extract was assayed directly for DMMPA by gas chromatography with a Varian 1440 instrument fitted with a Melpar flame photometric detector. The column was 1.8 m x 3.2 mm stainless steel packed with 3% OV 101 on 80/100 Chromosorb W. Operating conditions were :

Injection port temperature	270°C
Detector temperature	200°C
Column temperature	180°C
Carrier gas	Nitrogen, at 50 ml/min

The concentration of DMMPA in the hexane extract was determined from a calibration curve from appropriate standard solutions which were run on the same day. Internal standards were not required since no problems were experienced with emulsions (1).

For purity checks on DMMPA by gas chromatography, the column was 6.1 m x 3.2 mm stainless steel packed with 3% OV 17 on 80/100 HP Chromosorb G, and a flame ionisation detector was used. Operating conditions were :

Injection port temperature	220°C
Detector temperature	250°C
Column temperature	70° - 250°C at 6°C/min
Carrier gas	Nitrogen, at 15 ml/min

In some cases, the packing material was 10% UCW 98 on 80/100 mesh Diatoport S.

Animal experiments

Pure DMMPA (0.2-200 mg) in normal saline (total volume, 1.0 ml) was injected into the peritoneal cavity of adult hooded Wistar rats weighing 170-300 g. The rats were kept in a metabolism cage with water but no food, and urine was collected. At the end of set periods, the animals were killed by exsanguination after CO_2/O_2 (50:50) anaesthesia and the liver, kidneys and brain were removed whole and each weighed. Then a known wet weight of each organ (about 1 g) was homogenised in 7.5 ml water. For brain, the region extracted was mainly cerebral hemisphere. After homogenisation, the protein was precipitated with 1.5 ml tungstic acid solution and centrifuged (7). The aqueous extract was diluted with water to 10.0 ml, after noting the volume, and assayed for DMMPA as above. Aliquots of urine were diluted with water to 10.0 ml and also assayed for DMMPA.

In other experiments neat pure DMMPA (64 mg) was applied to the shaved back of a rat (200-235 g). After one hour, any DMMPA remaining on the skin was removed by wiping with small pieces of cotton wool, the first moistened with water and the second dry. The cotton wool was then suspended in 10 ml water and DMMPA extracted as described above. Urine was collected as above for 24 hours and also assayed for DMMPA.

Isolated organ experiments

Effects of DMMPA were determined on the isolated phrenic nerve-diaphragm preparation of the rat and the isolated right atrium of the guinea pig.

Thin layer chromatography

Merck pre-coated silicagel plates were developed in chloroform-methanol (9:1) and the spots made visible with iodine vapour. In some cases, zones of silicagel were removed from the plate and assayed for total phosphorus.

RESULTS

Analysis of DMMPA

Technical DMMPA contained a fine precipitate and most investigations were done on the supernatant. Limited investigations on the precipitate failed to reveal any organic material that was not present in the supernatant. Technical DMMPA was judged to be 85-90% pure by the results of gas chromatography, preparative thin-layer chromatography (t.l.c.) and column chromatography on silicagel. The sample contained < 0.1% inorganic phosphorus.

Gas chromatography revealed a total of eleven impurities in distilled technical DMMPA, although many of these were present in only trace amounts. Most of the major impurities eluted from the column before DMMPA, and were tentatively identified, by comparison with the elution behaviour of authentic samples, as N-methylmorpholine, morpholine and trimethylphosphate. Independent studies in Canada and U.S.A. have identified N-methylmorpholine and trimethylphosphate, as well as methyl chloride, as impurities of DMMPA (8,9). One significant peak of longer retention time than DMMPA was observed by the Canadian and American workers, and by us. The Canadians attribute this peak to the dimorpholine analogue of DMMPA, while the Americans propose a dimorpholine derivative of pyrophosphoric acid as the structure of their compound.

T.l.c. of technical DMMPA showed three impurities more polar than DMMPA. These do not correspond to any of the compounds listed above, with the possible exception of the dimorpholine compounds which were not available for comparison. Possible candidates for these impurities are dimethyl phosphate and the monomethyl analogue of DMMPA. One of the impurities does not appear to contain phosphorus and is possibly a morpholine derivative. The synthesized sample of DMMPA showed the same pattern of impurities on t.l.c. as did technical DMMPA.

Purification

Distillation under reduced pressure failed to purify DMMPA to a one-spot product on t.l.c. This objective could be achieved by filtration of DMMPA through Merck basic alumina in ether* (alumina: DMMPA = 2:1, w/w)

* We thank Defence Research Establishment, Suffield, for suggesting this procedure.

followed by fractional distillation under reduced pressure. The product was estimated by gas chromatography to be 99.8% pure.

Toxicity

The LD_{50} of purified DMMPA by intraperitoneal injection in mice was found to be approximately 4 g/kg. Coleman (2) reported a value of 5.3 g/kg. Technical DMMPA was 5-6 times more toxic than the purified material.

Assay for DMMPA

Many experiments were done in order to establish the efficiency of the assay procedure for DMMPA (see under Materials and Methods). Known amounts of DMMPA (0.01 - 20 mg, mostly 0.2 mg) were added to the aqueous phase before work-up. The results showed a considerable scatter and are presented as a histogram in Fig. 1. It is apparent that the extraction efficiency does not follow a normal distribution. It appeared to be independent of the nature of the aqueous phase i.e. whether it was water, urine or a tissue homogenate.

For the purpose of calculating recoveries of DMMPA in the animal experiments, the median value of the extraction efficiency (82%) was used.

Stability

As assessed by t.l.c., there was no decomposition of pure DMMPA stored over a desiccant at 2-5°C for 5 months. A sample stored at the same temperature, but not over a desiccant, did show signs of slight deterioration after this time. Another sample in 150 mM NaCl-5mM phosphate pH 7.4 was kept at 37°C for 3 days. Aliquots were assayed for DMMPA by gas chromatography during this period. Within the limits of the accuracy and reproducibility of the assay (above), no decomposition was observed. McNally and Adie (1) have reported that DMMPA is stable in refrigerated human urine under toluene for 6 months.

Persistence in the rat

DMMPA was injected into rats and its distribution determined after 4, 24 or 48 hours. The results for the tissues are given as recoveries in milligrams or micrograms in Table 1 and for the urine as a percentage of the administered dose in Table 2. When DMMPA was applied to the shaved back of a rat, the total percentage recovery in the urine in six experiments was 30, 17, 31, 15, 36 and 21%. One hour after application of 64 mg DMMPA, the amount of DMMPA which could be recovered from the skin by the cotton wool treatment was of the order of 1 mg or less. Absorption of DMMPA into the skin is therefore almost complete in this time.

Stability in human plasma

Since recovery from the rat is not quantitative, the possibility that DMMPA is hydrolysed by phosphatases in the blood was considered. Human plasma (0.5 ml), 32 mg DMMPA and 2.5 ml normal saline pH 7.4 were incubated at 37°C for 3 days. No decomposition of DMMPA was observed in duplicate experiments.

Effect on the neuromuscular junction and right atrium

The rat phrenic nerve-diaphragm preparation was exposed to DMMPA at concentrations ranging from 10^{-4}M to 5×10^{-3}M. Neither twitch tension at a stimulus rate of 0.5 Hz nor tetanic tension (5 s at 100 Hz) were altered by DMMPA for periods up to 50 min. The spontaneously beating guinea pig atrium was similarly insensitive to DMMPA, in spite of frothing with higher concentrations.

DISCUSSION

Excretion of DMMPA in the urine depends on the mode of administration. In man, DMMPA was administered percutaneously to two areas (fore-arm and cheek), intramuscularly, orally, and by inhalation of the vapour. The mean recoveries in the 24-hour urine were 2, 9-12, 17, 18 and 24% respectively (10,1,11). For the i.m., oral and inhalation routes a further 8% was excreted in the following 24 hours, and excretion in the urine continued for up to 4 days.

In the present work, i.p. injection of DMMPA into the rat caused DMMPA to appear in the 24-hour urine with a median recovery of 58% (n = 10). Very little DMMPA (mostly < 3% of the administered dose) was excreted in the next 24 hours. Topical application of DMMPA caused a lower recovery in the 24-hour urine; in this case the median (and mean) value was found to be 25% (n = 6).

Small amounts of DMMPA were found in the liver, kidney and brain at 4 hours after injection (Table 1) with the liver containing the highest amount, equal to 1.5 - 2.5% of the administered dose. This is consistent with the observation that material given by the intraperitoneal route in the rat is first passed through the liver (12). Much less DMMPA was found at 24 hours after injection (< 3% of 24-hour figures) and only trace amounts, i.e. microgram range, were recovered at 48 hours after injection of 200 mg DMMPA. These results assume a uniform distribution throughout the organ isolated. It should be noted that this dose of DMMPA (200 mg) is approximately 1/5th the LD_{50} as determined in mice. Apparently the organs of the rat are able to clear rapidly or metabolise DMMPA. Metabolism presumably accounts for the 40% of administered DMMPA that was not recovered over 48 hours, even though its degradation could not be demonstrated in human plasma.

We conclude that DMMPA does not accumulate in the body and is unlikely to present a chronic threat to the exposed animal or human.

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Dr. H.D. Crone did preliminary work on the extraction of DMMPA from tissues and gave advice on other aspects of the work. Dr. S.E. Freeman performed the isolated organ experiments. Dr. A.G. Moritz ran the n.m.r. spectra. Purity checks on DMMPA and investigation of impurities by gas chromatography were largely done by Mr. A.G. Kelso.

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TABLE 1

RECOVERY OF DMMPA FROM VARIOUS TISSUES OF THE RAT AFTER INTRAPERITONEAL INJECTION

The figures refer to extrapolated total amounts of DMMPA in each organ.

Organ	Weight (g)	DMMPA injected (mg)	Recovery at 4 h (mg)	Recovery at 24 h (μg)	Recovery at 48 h (μg)
Liver	5.3 - 7.4	2	0.02	0.7	0
	5.4 - 16.4	200	3.0 - 5.0 (n = 4)	52 - 96 (n = 4)	1 - 2 (n = 4), 10
Kidneys	1.3 - 1.5	2	0.01	0.3	0
	1.2 - 2.8	200	0.8 - 1.0 (n = 4)	12 - 22 (n = 4)	< 1 (n = 4), 3.5
Brain	1.8	2	0.015	0.4	0
	1.5 - 2.0	200	0.5 - 0.7 (n = 4)	15 - 21 (n = 4)	< 1 (n = 4), 1.4

TABLE 2

RECOVERY OF DMMPA FROM THE URINE OF THE RAT AFTER INTRAPERITONEAL INJECTION

DMMPA injected (mg)	Recovery (%)		
	0-4 h	0-24 h	24-48 h
0.2	23	67, 116	10.9
2	25	50, 56	1.0
200	10 - 21 (n = 4)	47 - 68 (n = 6)	0.5 - 3.1 (n = 4)

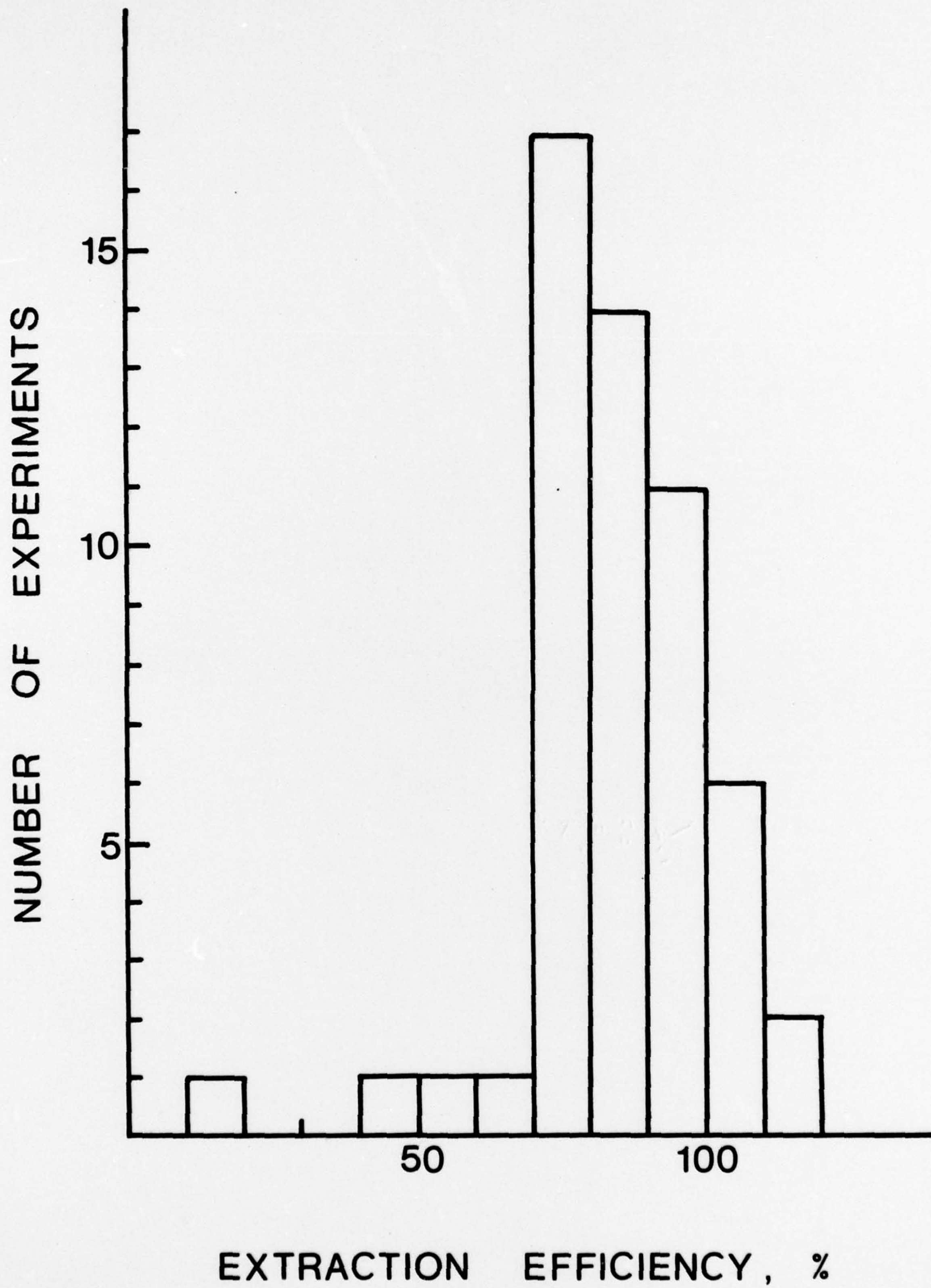

FIG. 1 - Efficiency of the procedure for extracting and assaying DMMPA from aqueous solution.