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Coexposure to Epichlorohydrin on the Elimination of Urinary Metabolites of Dimethylformamide

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N,N-Dimethylformamide (DMF) is an important industrial organic solvent. It is a well-known hepatotoxicant (Lundberg et al. 1981, Wang et al. 1991). Its reproductive effect is also documented in literature (Ducatman et al. 1986, Levin et al. 1987). Due to its low vapor pressure and high lipid solubility, the major occupational exposure route is through skin and mucous membrane (Lauwerys et al. 1980). In the past, the major occupational exposure to DMF in Taiwan occurred in the synthetic leather industry (Wang et al. 1991). Recently, however, results of occupational environmental monitoring have revealed that the newly evolved multi-billion-dollar electronics industry also uses large volume of DMF. A direct-acting mutagen, epichlorohydrin (ECH, 1,2-epoxy-3-chloropropane), is also heavily used in the electronics industry. It is used in the production of epoxy-resins and printed circuit board. In manufacturing of the latter, DMF is also used.

ECH is a proven animal carcinogen (Konishi et al. 1980, Laskin et al. 1980, Webster et al. 1985), and a reproductive toxicant (Milby et al. 1981, Toth et al. 1991). Its exposure route is both via inhalation and skin absorption. The reproductive effect of both DMF and ECH is on male testicular function. The *in vivo* metabolism of ECH has been reported as partly goes through a transformation by epoxide hydrase to alpha-chlorohydrin (ACH). And most of the ACH is further metabolized to CO_2 (Gingell et al. 1985). The other part of the *in vivo* metabolism involves a direct conjugation with glutathione (GSH) to form N-acetyl-S-(3-chloro-2-hydroxypropyl) cysteine (ACPC), which is then eliminated in urine (Gingell et al. 1985, DeRooij et al. 1996). On the other hand, the initial *in vivo* metabolism of DMF involves an alpha-hydroxylation yielding a thermally and alkaline unstable metabolite, N-methyl, N-hydroxymethyl-formamide (DMF-OH) (Scailteur and Lauwerys 1984). Part of the DMF-OH formed also conjugates with GSH to yield N-acetyl-S-(N-methylcarbamoyl) cysteine (AMCC), which is excreted in urine as well (Marz and Turecek 1987).

Conventionally, the biological monitoring of occupational exposure to DMF has been by analysis of urinary N-methyl-formamide (NMF), which mainly results from the thermal decomposition product of DMF-OH occurring at the injection port of a gas chromatograph (GC) (Krivanek et al. 1978, Lauwerys et al. 1980, Kawai et al. 1992). Indirect measurement of urinary DMF-OH has also been used

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to show its linear relationship with DMF doses given to rats by Chang and Lin (1991). It has also been demonstrated that excessive exposure to DMF would result in an excretion of DMF itself in urine (Lareo and Perbellini 1995).

In this report, we have taken a toxicological approach to investigating the effect of co-exposure to ECH in rats on the elimination of urinary metabolites of DMF, namely DMF-OH, NMF, and DMF. To confirm the experimental findings, we have also evaluated the effect of ECH treatment on the tissue level of GSH in liver, kidney, and testis. Both testis and kidney are the toxic target organs of ECH (Milby et al. 1981, Boogaard et al. 1993).

MATERIALS AND METHODS

To evaluate the effect of co-exposure to ECH on the urinary elimination of DMF and its metabolites, adult male Wistar rats were used. Rats were housed individually in metabolic cages. Four animals per dose group were orally given ECH (99.8% certified, Tedia Co., Fairfield, OH) dissolved in Mazola corn oil. The dosages evaluated were 0, 4.5, 9.0, and 18.0 mg/kg b. wt., which were about the dosages studied by Gingell et al. (1985) and de Rooij et al. (1996). Within 30 minutes, the rats were also gavaged with DMF (99.8%, Merck, Darmstadt, FRG) dissolved in deionized water at 72 mg/kg b. wt., which was close to the lower end of the range evaluated by Chang and Lin (1991). Individual urine samples were collected in the dark for 24 hours following treatment. The volume and pH of each urine sample were recorded. Each sample was further allocated into several 2-mL Eppendorf tubes and stored at -30C until its analysis for urinary metabolites.

Analyses of urinary NMF and DMF were done together by capillary GC (DB-624, 0.53 mm x 30 m, 3.0 μ) coupled with flame ionization detection (FID). An internal standard, N-methyl acetamide (NMA, 97%, Fluka Chemika, Switzerland), was included in the analysis. Two independent calibration curves were established for NMF (>99%, Merck, Darmstadt, FRG) and DMF with each batch of the analyses. The recovery of NMF-spiked urine samples was 100 ± 10% (n=4) and DMF-spiked, 99 ± 8% (n=4). Sample preparation involved a quick thaw of an aliquot of the frozen urine at 37C, a brief centrifugation to remove particulate matters, denaturation with 2-volume of cold methanol containing the internal standard, and another brief centrifugation to remove the denatured protein before the instrumental analysis. Before denaturation, an aliquot of the supernatant was saved for the determination of urinary creatinine, which was determined by the Jaffe method. Urinary DMF-OH was analyzed, freshly after collection, according to the Chang and Lin method (1991).

To evaluate the effect of ECH treatment on the tissue level of reduced-GSH, again male Wistar rats were used. Rats, three per dose group, were gavaged with ECH dissolved in Mazola corn oil and two studies were performed. Initially, the dosages used were identical to the above co-exposure study, i.e. 0, 4.5, 9.0, and 18.0 mg/kg b. wt., and the rats were treated for 24 hours before sacrifice. For the

repeat study, the dosages were increased to 0, 9.0, 18.0, and 36.0 mg/kg b. wt., and the treatment went on for 4 hours only. Testes, kidneys, and livers were harvested at the time of sacrifice and immediately frozen in liquid nitrogen and kept at -70C until analysis. The analysis of reduced-GSH was done essentially by the Mokrasch and Teschke method (1984), as described in detail in the report of Chang et al. (1993). Statistical comparisons among dose groups were done by ANOVA.

RESULTS AND DISCUSSION

Summarized in Table 1, 2, and 3 are the urinary DMF-OH, NMF, and DMF of each individual rat, and the group means and standard errors of mean. Statistically, there is no significant difference among the various ECH dose groups, even though there is potential competition for GSH between the detoxification of DMF and ECH via a conjugation with it. Clearly, no ECH dose response relationship was observed on the elimination of the metabolites of DMF.

Table 1. Effect of co-exposure to ECH on the first day urinary elimination of DMF-OH in µg/mL

ECH, mg/kg					Mean ± sem
0	506	344	863	826	635 ± 126
4.5	547	515	603	1104	692 ± 139
9.0	505	960	993	882	835 ± 113
18.0	332	655	562	591	535 ± 70

Table 2. Effect of co-exposure to ECH on the first day urinary elimination of NMF in µg/mL

ECH, mg/kg					Mean ± sem
0	838	429	1600	1571	1110 ± 287
4.5	865	980	1028	2060	1233 ± 278
9.0	907	2517	1778	1551	1688 ± 332
18.0	581	1725	1007	1042	1089 ± 237

Table 3. Effect of co-exposure to ECH on the first day urinary elimination of DMF in µg/mL

ECH, mg/kg					Mean ± sem
0	319	214	626	699	465 ± 118
4.5	187	408	653	956	551 ± 165
9.0	409	580	616	692	574 ± 60
18.0	262	554	439	494	437 ± 63

An alpha-hydroxylation of DMF by liver microsomal enzyme to form DMF-OH was the very first step in the metabolism of DMF (Scailteur and Lauwerys 1984). Due to its instability, DMF-OH standard was not commercially available. Its

analysis was therefore proceeded by an indirect measurement as described by Scailteur and Lauwerys (1984) and Chang and Lin (1991). Data were presented in Table 1. Chang and Lin have shown that the DMF-OH measured by this means demonstrated a linear dose response relationship in rats, ranging from 47.2 to 944 mg/kg b. wt. The thermally decomposed product, NMF (Table 2), was determined by capillary GC/FID. Based on equal molar transformation of DMF-OH to NMF, the values in Table 2 are much greater than the corresponding data shown in Table 1. This is compatible with the observation of Scailteur and Lauwerys in 1984, that demethylation of DMF to NMF does occur *in vivo.*

In the field of occupational hygiene practice, the biological monitoring of exposure to DMF is to measure urinary NMF, either in µg/mL or µg/mg creatinine. In this report, we have also determined the concentration of each individual urinary creatinme by the Jaffe reaction. The results are presented in Table 4. Again, an exposure to ECH seems do not affect the basal elimination of creatinme. We have also evaluated the DMF-OH, NMF, and DMF determined in µg/mg creatinine, and found that the conclusion remains the same.

Table 4. Effect of co-exposure to ECH on the first day concentration of urinary creatinine of the DMF treated rats in mg/mL

ECH, mg/kg					Mean ± sem
0	0.778	0.474	1.230	1.187	0.917 ± 0.180
4.5	0.679	1.107	0.856	1.612	1.064 ± 0.203
9.0	0.920	1.574	1.220	1.318	1.258 ± 0.135
18.0	0.614	1.413	0.687	0.726	0.860 ± 0.186

In terms of supporting the hypothesis that a metabolic competition of ECH for GSH might affect the urinary elimination of DMF and its metabolites, the negative results we've obtained become circumstantial. One of the possible definitive studies would be an investigation of a co-exposure to ECH on the elimination of AMCC, the resultant conjugate of DMF-OH with GSH. However, the analysis of AMCC requires more sophisticated instrumentation, i.e. GC coupled with a MSD (mass spectral detection). We have decided to take a different approach, to investigate the effect of ECH on the tissue levels of reduced-GSH. We have not only measured the GSH levels in liver, the major metabolic organ but also the target organs of ECH, i.e. kidney and testis. Both testis and liver are also the target organs of DMF.

Initially, the ECH dosages evaluated were identical to the above co-exposure study, namely 0, 4.5, 9.0 and 18.0 mg/kg b. wt., and the treatment was also for 24 hours. The results are presented in Table 5. Essentially, there is no difference in tissue levels of GSH among the various dose groups. Since ECH is a direct acting mutagen, it was reasoned that the 24-hour treatment might be too long. A partial repeat study was undertaken with a higher dose group, 36.0 mg/kg b. wt., and the animals were treated for 4 hours only. The results are presented in Table 6. The same conclusion is drawn; that a treatment of various dosages of ECH, up to 36.0

Table 5. Effect of ECH on the tissue level of GSH in Male Rats, (mean ± sem in nmol/mg protein), measured at 24 hours after the treatment

ECH, mg/kg	Testis	Kidney	Liver
0	7.85 ± 0.39	1.49 ± 0.16	6.94 ± 0.65
4.5	8.07 ± 0.39	1.52 ± 0.14	7.45 ± 0.72
9.0	8.08 ± 0.46	1.47 ± 0.13	6.79 ± 0.15
18.0	7.91 ± 0.44	1.43 ± 0.17	6.99 ± 0.46

Table 6. Effect of ECH on the tissue level of GSH in male rats (mean ± sem in nmol/mg protein), measured at 4 hours alter the treatment

ECH, mg/kg	Testis	Kidney	Liver
0	9.47 ± 0.21	1.68 ± 0.08	7.75 ± 0.69
4.5	9.58 ± 1.26	1.61 ± 0.09	8.42 ± 0.50
9.0	9.84 ± 0.50	1.66 ± 0.16	8.84 ± 0.71
18.0	9.32 ± 0.67	1.66 ± 0.17	8.94 ± 0.32

mg/kg b. wt., did not modify the tissue levels of GSH. One of the possible explanations is that ECH is such an active, direct acting electrophile that most of the dose will react immediately with the abundantly available nucleophilic centers of all of the biological macromolecules *in vivo*. This overwhelming number of nucleophilic centers simply dilutes out the effect of ECH on tissue levels of GSH.

Based on these results, we have concluded that an exposure to ECH does not significantly affect the tissue level of reduced-GSH, nor does it modify the urinary elimination of DMF and its metabolites.

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