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S-(N-METHYLCARBAMOYL)GLUTATHIONE : A REACTIVE S-LINKED METABOLITE OF METHYL ISOCYANATE

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Summary : S-(N-methylcarbamoyl)glutathione, a chemically-reactive glutathione conjugate, has been isolated from the bile of rats administered methyl isocyanate and characterized, as its N-benzyloxycarbonyl dimethylester derivative, by tandem mass spectrometry. The ability of this glutathione adduct to donate an N-methylcarbamoyl moiety to the free -SH group of cysteine was evaluated *in vitro* with the aid of a highly specific thermospray LC/MS assay procedure. The glutathione adduct reacted readily with cysteine in buffered aqueous media (pH 7.4, 37°C) and after 2 hr, 42.5 % of the substrate existed in the form of S-(N-methylcarbamoyl)cysteine. The reverse reaction, i.e. between the cysteine adduct and free glutathione, also took place readily under these conditions. It is concluded that conjugation of methyl isocyanate with glutathione *in vivo* affords a reactive S-linked product which displays the potential to carbamoylate nucleophilic amino acids. The various systemic toxicities associated with exposure of animals or humans to methyl isocyanate could therefore be due to release of the isocyanate from its glutathione conjugate, which thus may serve as a vehicle for the transport of methyl isocyanate *in vivo*.

Methyl isocyanate (MIC) , an important intermediate in the synthesis of a variety of industrial products, is a highly toxic vesicant and irritant. In the aftermath of the Bhopal incident, in which MIC was released into the atmosphere, considerable attention has focused upon the pathophysiological changes associated with human exposure to this compound (1). Important clinical features in survivors of the disaster were eye and respiratory tract irritation (2) and it has been reported that some 10% of the exposed population displayed pathological changes in the lung associated with emphysema (3). The pulmonary pathology (4,5), peripheral emphysema and severe ocular irritation (6) induced by MIC have been reproduced in animal models. Somewhat surprisingly, in view of the high chemical reactivity of MIC, *systemic* toxicities have been observed in organs remote from the primary site of exposure, and have included infertility (7) and myelotoxicity (8). Interestingly, inhalation studies in

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ABBREVIATIONS : CID, collision induced dissociation; CYS, cysteine; FAB/MS, fast atom bombardment mass spectrometry; GSH, glutathione; HPLC, high-performance liquid chromatography; MIC, methyl isocyanate; MS-MS, tandem mass spectrometry; SMC, S-(N-methylcarbamoyl)cysteine; SMG, S-(N-methylcarbamoyl)glutathione; TSP-LC/MS, thermospray liquid chromatography mass spectrometry.

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rodents exposed to [^{14}C]MIC have demonstrated a rapid uptake and systemic distribution of radiolabel to extrapulmonary organs (9). Moreover, covalent modification of hemoglobin (10) and hepatocellular proteins (11) has been observed following exposure to MIC vapor. However, the chemical nature of this systemically distributed MIC is unclear, although it has been speculated that pulmonary conjugation of MIC with GSH may play a role in the transport of this toxic chemical (9). By analogy, a biliary metabolite of the investigational anti-tumour agent N-methylformamide (NMF) has been identified as S-(N-methylcarbamoyl)glutathione (SMG), a carbamate thioester conjugate which is believed to be formed *via* the intermediacy of metabolically-generated MIC (12). The structural similarities between SMG and reactive glutathione and cysteine conjugates of allyl- and benzyl isothiocyanate (13), which release free isothiocyanate under physiological conditions, suggests that SMG may well release MIC in a rapid reversible manner. According to this scenario, SMG, formed as a metabolite of MIC, may thus serve as a latent carrier to deliver MIC to extra-pulmonary organs where toxicities have been observed.

The goals of the preliminary study described in this communication were to test the hypotheses that (i) MIC does form SMG *in vivo*, and (ii) this GSH conjugate acts as a carbamoylating agent towards nucleophilic acceptors, e.g. CYS, consistent with its proposed role as a biological transport vehicle for MIC.

MATERIALS AND METHODS

Synthesis: S-(N-methylcarbamoyl)glutathione (SMG), S-(N-methylcarbamoyl)cysteine (SMC), S-(N-ethylcarbamoyl)cysteine (SEC) were prepared by a novel synthetic procedure which is described in detail elsewhere [14].

Administration of MIC and isolation of SMG from bile: Two male Sprague-Dawley rats (250g ; Charles River Laboratories, Wilmington, MA) were anaesthetized, the bile duct of each was cannulated and MIC (12 mg/kg) in DMSO (50 µl) was infused into the portal vein. Bile was collected for 1 hr and metabolites were derivatized with benzylchloroformate and anhydrous methanolic hydrogen chloride and purified by reversed-phase HPLC as described previously (15). In a seperate experiment two male Sprague-Dawley rats (250g) were maintained under anaesthesia and received i.p. injections of MIC (7.5 mg/kg) in DMSO (50 µl) at hourly intervals during the course of a four hour bile collection period. The bile from these animals was derivatized and SMG was isolated as described above.

Carbamoylation of cysteine by SMG: SMG (1 mM) was incubated with cysteine (5 mM) in 100 mM aqueous phosphate buffer (pH 7.4) at 37 °C and aliquots were removed at various time points. To each aliquot was added acidic mobile phase (see below) containing 0.5 mM SEC as internal standard, and the products were analyzed by LC/MS. SMG and SMC were quantified relative to SEC by selected ion recording of ions at m/z 179 (SMG) and 163 (SMC, SEC). The reverse reaction (i.e. between SMC and GSH) was carried out similarly.

Mass Spectrometry: Conventional FAB spectra and CID daughter ion spectra were recorded on a VG 70-SEQ hybrid tandem instrument of EBQQ geometry, equipped with an Ion-Tech fast atom gun and a VG11/250 data system. CID was performed in the first (rf-only) quadrupole employing argon as a collision gas (2 x 10^{-6} Torr) and a collision energy of 35 eV. LC/MS was carried out on a Vestec Model 201 thermospray LC/MS system equipped with a Hewlett-Packard 5997 ChemStation data system. The mobile phase was 2.5 % acetonitrile/ 97.5 % 50 mM NH_4Ac (pH 4.5, 0.75 mL/min) and separations were carried out on an Altex Ultrasphere 5 µm C_{18} column (15 cm x 4.6 mm). Post-column addition of 80 % acetonitrile/H_2O (1 mL/min) served to optimize sensitivity of detection of the conjugates-of-interest.

RESULTS AND DISCUSSION

Characterization of S-(N-methylcarbamoyl)glutathione (SMG) as a biliary metabolite of methyl isocyanate: Bile from rats dosed with MIC by either the intraportal or intraperitoneal route was processed by the two-step derivatization and HPLC purification sequence developed for use with GSH conjugates of N-alkylformamides (12,15). Following the first derivatization step (reaction with benzylchloroformate), HPLC analysis of these bile extracts revealed the presence of a component with the same retention time (9.8 min) as N-benzyloxycarbonyl SMG. This material was collected and converted to the corresponding dimethyl ester derivative which, upon subsequent HPLC analysis, was found to co-chromatograph with authentic N-benzyloxycarbonyl SMG dimethyl ester. This latter derivative was isolated and subjected to analysis by FAB/MS and FAB/MS/MS. Conventional FAB/MS of the derivatized conjugate revealed a prominent ion at m/z 527 (Figure 1A), corresponding to the MH^+ of the N-benzyloxycarbonyl dimethyl ester of SMG, and was accompanied by an $[M+Na]^+$ adduct ion at m/z 549. A daughter ion spectrum (CID of m/z 527) of the derivatized metabolite (Figure 1B) was identical to that recorded form the synthetic reference standard (Figure 1C). In each of the CID spectra, structurally-informative daughter ions were observed indicative of the γ-glutamyl- (ion **e**), cysteinyl- (ion **g**) and glycyl- (ion **a**) components of the conjugate; the mass spectral fragmentation of derivatized SMG under MS/MS conditions is discussed in detail elsewhere (15).

Carbamoylation of free thiols by SMG and SMC *in vitro*: Upon incubation of SMG with cysteine in aqueous solution at pH 7.4, S-(N-methylcarbamoyl)cysteine (SMC) was identified as the major product by TSP-LC/MS. The rates of formation of SMC and consumption of SMG were quantified as described previously (16). A steady decline in the concentration of SMG (initial $t_{1/2}$ = 90 min) was paralleled by an increase in the amount of SMC formed (Figure 2). The reaction reached equilibrium in 2 hr, at which time SMC represented 42.5 % of total remaining carbamate thioesters (SMG + SMC). After 3 hr, the concentration of total carbamate thioesters indicated a net loss of 31.5 % of the SMG present at the outset (initial $t_{1/2}$ = 292 min). This value was comparable to the total amount of SMG lost (hydrolyzed) in a control incubation which contained SMG alone (initial $t_{1/2}$ = 264 min). Figure 3 shows the time-dependent loss of SMC (initial $t_{1/2}$= 55 min) and concomitant formation of SMG from the reaction between SMC and GSH. The enhanced rate of disappearance of SMG in the presence of added thiol, and the reversible nature of the reaction, agree well with the observations of Bruggeman *et al.* [13] on the behavior of CYS and GSH conjugates of isothiocyanates in aqueous media.

Based on the known chemical behavior of secondary carbamates in aqueous media (17), it appears likely that the exchange reaction between SMG or SMC and free thiols proceeds according to an E_1cB mechanism (unimolecular elimination *via* the conjugate base). According to this mechanism, proton abstraction from nitrogen results in the formation of a conjugate base which then eliminates the thiol portion of the carbamoyl thioester and releases MIC. The more rapid rate of reaction observed for the cysteine conjugate and GSH (Figure 3) relative to that found for SMG and cysteine (Figure 2) has also been noted in the exchange reactions of isothiocyanates (13) and correlates with the relative stabilities of the cysteine and glutathione conjugates in buffer. The enhanced reactivity of SMC relative to SMG may be rationalized by invoking a mechanism in which proton abstraction from the carbamate

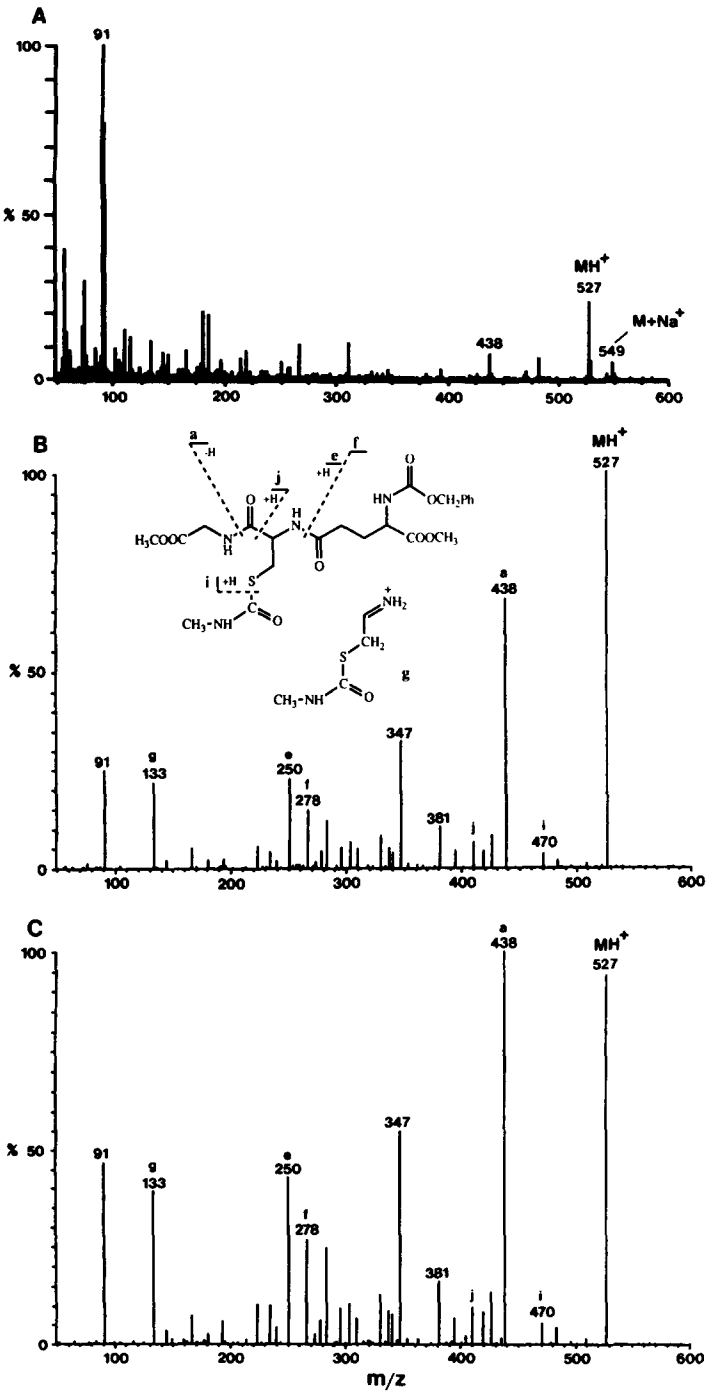
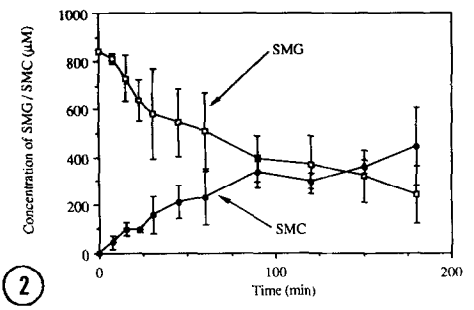


Figure 1. FAB mass spectra of derivatized samples of SMG. (A) Conventional spectrum of a derivatized extract of bile collected from a rat dosed with methyl isocyanate. The MH^+ and $M+Na^+$ ions from the SMG derivative are evident at m/z 527 and 549, respectively. (B) Daughter ion spectrum obtained by CID of the MH^+ ion (at m/z 527) in the spectrum shown in (A). The structure of the metabolite and proposed origins of the daughter ions is as indicated. (C) Daughter ion spectrum obtained by CID of the MH^+ ion (at m/z 527) of an authentic sample of derivatized SMG.



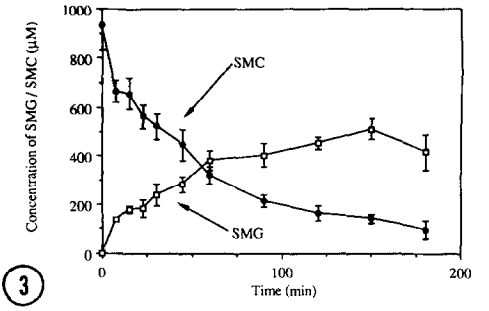


Figure 2. Carbamoylation of CYS by SMG. SMG (1 mM) was incubated with CYS (5 mM) in phosphate buffer (pH 7.4 ; 100 mM). Aliquots were removed at various time points and SMC and SMG were quanitfied by TSP / LC-MS. The results are expressed as means ± S.D. of 3 determinations.

Figure 3. Carbamoylation of GSH by SMC. SMC (1 mM) was incubated with GSH (5 mM) in phosphate buffer (pH 7.4 ; 100 mM). Aliquots were removed at various time points and SMC and SMG were quanitfied by TSP / LC-MS. The results are expressed as means ± S.D. of 3 determinations.

nitrogen occurs by intramolecular transfer to the cysteine carboxylate anion, thereby facilitating E_1cB-mediated elimination of MIC from this conjugate. The time-dependent decline in the concentration of S-linked conjugates observed in the present studies may be accounted for by partial hydrolysis of MIC to N-methylcarbamic acid which, in turn, undergoes spontaneous decomposition to carbon dioxide and methylamine (18).

CONCLUSIONS

The results from this preliminary study have shown that when MIC is administered parenterally to rats (by either intraportal or intraperitoneal injection), a glutathione conjugate (SMG) is formed whose structure is identical to that generated during metabolic activation of NMF (12). This finding provides indirect support for the contention that MIC is the reactive, hepatotoxic intermediate produced during the biotransformation of NMF in animals and human subjects (12). In addition, these results have important implications with respect to the consequences of pulmonary exposure to MIC in man as appreciable levels of GSH are present in the epithelial lining fluid of the lower respiratory tract (19). Thus, it may be surmised that inhaled MIC would be converted, at least in part, to SMG in the lung, and that a portion of this adduct would be absorbed into the systemic circulation (9). In light of our own observation that SMG itself is a chemically-reactive species which carbamoylates free sulfhydryl groups (and possibly other nucleophilic centers on biological molecules) under physiological conditions, SMG would appear to be an attractive candidate for the "transport" form of MIC *in vivo*. In this context, conjugation of MIC with GSH would lead not simply to detoxification of the isocyanate, but rather to an extension of the adverse effects of MIC to organ systems not exposed directly to this toxic chemical. Studies to examine this intriguing possibility are currently in progress.

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REFERENCES

1 Bucher, J.R. (1987) *Fund. Appl. Toxicol.* **9**, 367-379.

2 Misra, U.K., Nag, D., Nath, P., Khan, W.A., Gupta, B.N., Seth R.K., Dwivedi, R.S. and Ray, P.K. (1988) *Indian J. Exp. Biol.* **26**, 201-204.

3 Gupta, B.N., Rastogi, S.K., Chandra, H., Mathur, N., Mahendra, P.N., Pangtey, B.S., Kumar, S., Seth R.K., Dwivedi, R.S. and Ray, P.K. (1988) *Indian J. Exp. Biol.* **26**, 149-160.

4 Ferguson, J. S., Schafer, M., Stock, M.F., Weyel, D.A. and Alarie, Y. (1986) *Toxicol. Appl. Pharmacol.* **82**, 329-335.

5 Alarie, Y., Ferguson, J. S., Stock, M.F., Weyel, D.A. and Schafer, M. (1987) *Environ. Health Perspect.* **72**, 159-167.

6 Dutta, K.K., Gupta, G.S.D., Mishra, A., Joshi, A., Tandon, G.S. and Ray, P.K. (1988) *Indian J. Exp. Biol.* **26**, 177-182.

7 Varma, D.R., Ferguson, J.S. and Alarie, Y.J. (1987) *Toxicol. Environ. Health.* **21**, 265-275.

8 Conner, M.K., Rubinson, H.F., Ferguson, J.S., Stock, M.F. and Alarie, Y. (1987) *Environ. Health Perspect.* **72**, 177-182.

9 Ferguson, J.S., Kennedy, A.L., Stock, M.F., Brown, W.E. and Alarie Y. (1988) *Toxicol. Appl. Pharmacol.* **94**, 104-117.

10 Ramachandran, P.K., Gandhe, B.R., Venkateswaran, K.S., Kaushik, M.P., Vijayaraghavan, R., Agarwal, G.S., Gopala, N., Suryanarayana, M.V.S. and Shinde, S.K. (1988) *J. Chromatogr.* **426**, 239-247.

11 Bhattacharya, B.K., Sharma S.K. and Jaiswal, D.K. (1988) *Biochem. Pharmacol.* **37**, 2489-2493.

12 Threadgill, M.D., Axworthy, D.B., Baillie, T.A., Farmer, P.B., Farrow, K.C., Gescher, A., Kestell, P., Pearson, P.G. and Shaw, A.J. (1987) *J. Pharmacol. Exp. Ther.* **242**, 312 - 319.

13 Bruggeman I.M., Temmink J.H.M. and van Bladeren, P.J. (1986) *Toxicol. Appl. Pharmacol.* **83**, 349 - 359.

14 Han D.-H., Pearson, P.G. and Baillie, T.A. (1989) *J. Labelled Comp. Radiopharm.* (in press)

15 Pearson, P.G., Threadgill, M.D., Howald, W.N. and Baillie T.A. (1988) *Biomed. Environ. Mass Spectrom.* **16** , 51-56.

16 Rashed, M.R., Pearson, P.G., Han, D.-H. and Baillie, T.A. (1989) *Rapid Commun. Mass Spectrom.* **3**, 360 - 363.

17 Dittert, L.W. and Higuchi, T. (1963) *J. Pharm. Sci.* **52**, 852 - 857.

18 D' Silva, Lopes, A., Jones, R.L., Singhawangha, S. and Chan, J.K. (1986) *J. Org. Chem.* **51**, 3781-3788.

19 Cantin, A.M., North, S.L., Hubbard, R.C. and Crystal, R.G. (1987) *J. Appl. Physiol.* **63**, 152-157.