Assessment of occupational exposure to 4,4'-diaminodiphenylmethane (methylene dianiline) by gas chromatography-mass spectrometry analysis of urine

J COCKER, W GRISTWOOD, H K WILSON

From the Occupational Medicine and Hygiene Laboratories, Health & Safety Executive, London NW2 6LN, UK

ABSTRACT A new specific and sensitive method has been used to monitor workers from five different factories where 4,4'-diaminodiphenylmethane (methylene dianiline) (DDM) was being used. The isolation and identification of an N-acetyl conjugate of DDM, a major metabolite of DDM found in human urine, is reported for the first time. The use of this biological monitoring method will allow the assessment of the absorption of DDM and help in monitoring improvements in work practices, particularly where exposure may occur through pathways other than inhalation.

4,4' Diaminodiphenylmethane (DDM) is a commercially important aromatic amine used in the production of isocyanates, polyisocyanates, polyurethane foams, and elastomers and as an epoxy resin hardener.¹ It is commonly referred to as methylene dianiline (MDA) (CAS 101-77-9). Several workers have studied the toxicity of DDM. It has been reported to be hepatotoxic in rats,^{2,3} dogs,⁴ and man.⁵⁻⁸ It is mutagenic in the Ames test in the presence, but not in the absence, of an S9 metabolising system⁹ and has been reported to be carcinogenic in rats and mice.^{10,11} As a result of these findings and the close chemical similarity of DDM to other aromatic amines, which are proved or suspect human carcinogens, DDM is under scrutiny by regulatory bodies in the United States.

Occupational exposure to DDM may be assessed by measuring DDM concentrations in the work environment and by biological monitoring. DDM, like 3,3'-dichloro 4,4'-diaminodiphenylmethane (commonly known as 4,4' methylene bis 2-chloroaniline or MBOCA) and other aromatic amines, has the potential for considerable skin absorption. Analysis of urine samples from workers exposed to MBOCA is helpful in assessing absorption and in monitoring improvements in occupational hygiene.¹² Analytical methods have been reported for the analysis of DDM in workplace air using high performance liquid chromatography (HPLC) with ultraviolet detection¹³ and in blood using gas chromatography (GC) with elec-

tron capture detection.¹⁴ The work reported here describes the use of a new sensitive and specific gas chromatographic-mass spectrometric (GCMS) method for measuring DDM in urine. This approach was adopted after we had used HPLC and GC methods and had noted that occasionally interfering peaks were present in urine. We also describe for the first time the isolation and identification of N-acetyl DDM, a major metabolite of DDM found in human urine. These findings are used to devise a biological monitoring method for monitoring workers exposed to DDM.

Materials and methods

URINE SAMPLES

Urine samples were collected from 111 workers at five factories using DDM. Factories A and B were packaging DDM as a component of a hardener in a two part epoxy resin formulation, factories C and D were using DDM and other compounds to impregnate carbon fibre mats, and factory E used DDM as a component in a screen printing process for seals and gaskets. Urine samples were collected in polycarbonate bottles towards the end of a working shift and dispatched to the laboratory where they were stored at -20°C and analysed within five days.

MEASUREMENT OF TOTAL DDM IN URINE

Procedure

Urine (2 ml) was placed in a 20 ml glass tube and 10 µmol/l MBOCA solution (100 µl) was added as an

Accepted 3 March 1986.

Fig 1 Electron impact mass spectra of the diheptafluorobutyl derivatives of (a) DDM and (b) MBOCA.

internal standard. After adding 10 M sodium hydroxide (2 ml) (pH13) the tube was heated in a water bath at 80°C for 60 minutes then cooled to room temperature. DDM and MBOCA were extracted into diethyl ether (10 ml) using a rotary tumbler (10 min). The organic layer was removed and evaporated using a vortex evaporator and the residue was derivatised by adding 1% heptafluorobutyl chloride in cyclohexane (1 ml) and heating in a dry block (90°C, 40 min). The excess reagent was removed by evaporation and the sample was dissolved in toluene (100 µl). The solution (1 µl) was injected into the GCMS. The molecular ion of DDM (m/z 590) (fig 1a) and a fragment ion (M-Cl, m/z 623) of MBOCA (fig 1b) were monitored. The concentration of DDM in the sample was calculated from a calibration curve obtained from known concentrations of DDM. This curve was linear from 20 nmol/l to at least 1500 nmol/l.

The optimum pH for extraction of DDM and MBOCA from urine has been reported previously and the recovery of DDM from urine is greater than 85%.¹⁵ Typical selected ion chromatograms from occupationally exposed and non-exposed workers' urine taken through the analytical procedure are shown in figs 2a and 2b respectively. Results reported

from this laboratory are adjusted for urinary creatinine concentrations and are expressed as nmol DDM/mmol creatinine. Daily variation in the method was monitored by a quality control scheme. Quality control material was prepared from urine collected from a volunteer not exposed to DDM and was

Fig 2 Typical selected ion chromatograms from urine of (a) a worker exposed to DDM and (b) an unexposed worker. Ions at m/z 590 from DDM-HFB and at m/z 623 from MBOCA-HFB. Peak 1 = DDM, 2 = MBOCA.

fortified with N-acetyl DDM to give a final concentration of about 200 nmol/l and stored at -20°C . The coefficients of variation for within batch and day to day analysis were approximately 5% and 9% respectively.

Instrumentation

All analyses were performed on a Carlo Erba 5160 gas chromatograph, fitted with a $25\text{ m} \times 0.2\text{ mm}$ fused silica column coated with BP1. The gas chromatograph was interfaced to a VG analytical 16F mass spectrometer linked to a VG 2035 data system. The initial oven temperature was 120°C for the injection period. The oven temperature was then increased at $30^{\circ}/\text{min}$ to 280°C and held at this temperature for four minutes.

DETERMINATION OF THE EXTENT OF HYDROLYSIS

An experiment was carried out to determine whether the procedure described above was effective in releasing DDM from possible metabolites such as N-acetyl DDM, N,N'-diacetyl DDM, and N-glucuronides. Urine from three workers exposed to DDM was subjected to mild and strong hydrolysis. The strong hydrolysis procedure is described above. Mild hydrolysis entailed omitting the sodium hydroxide and subjecting the urine to heat alone. Samples were removed at timed intervals for analysis. To determine the efficiency of hydrolysis of N-acetyl and N,N'-diacetyl DDM, the synthetic material (see below) was added to a urine sample from an unexposed person and the samples were taken through both hydrolysis procedures.

ISOLATION OF N-ACETYL DDM FROM URINE

Aliquots of urine (1 ml) from workers exposed to DDM were injected on to a $25\text{ cm} \times 4\text{ mm}$ C18 HPLC column. The mobile phase was varied using a non-linear gradient from 100% water to 100% methanol with a flow rate of 2 ml/min over 30 minutes. Fractions were collected at one minute intervals. The fractions with an elution volume similar to pure N-acetyl DDM were subjected to off-line mass spectrometry using a direct insertion probe.

SYNTHESIS OF N-ACETYL AND N,N'-DIACETYL DDM

N-acetyl DDM and N,N'-diacetyl DDM were synthesised by adding 70 mmol acetic anhydride slowly to a solution of 50 mmol DDM in 10 ml of pyridine. After heating for 30 minutes at 80°C the reaction mixture was poured into distilled water, the resulting precipitate was filtered, washed with more distilled water, and then dried. The products were separated and purified by HPLC on a silica column using 4% meth-

anol in dichloromethane. The identities of the products were confirmed by electron impact mass spectrometry. N-acetyl DDM gave a spectrum with a base peak and molecular ion at m/z 240 and significant fragment ions at m/z 197 (75%), 106 (56%), 198 (52%), 182 (25%), and 43 (23%). N,N'-diacetyl DDM gave a spectrum with a molecular ion and base peak at m/z 282 and significant fragment ions at m/z 240 (78%), 198 (71%), 197 (65%), 106 (56%), 43 (51%), and 182 (29%).

Results

Preliminary investigations carried out in this laboratory indicated that GC or HPLC methods may be unsuitable for investigating the excretion of DDM and its metabolites in the urine of exposed workers. With these methods peaks are occasionally found in some urine samples which co-elute with DDM. This prompted us to develop a more specific and sensitive GCMS method for measuring DDM in urine for use in the study of DDM metabolism and excretion in an occupationally exposed population.

During a 10 month period, over 300 urine samples have been analysed from five different factories using DDM. The frequency distribution in fig 3 shows that most (81%) of these samples contained less than 5 nmol DDM/mmol creatinine—that is, below the detection limit of 50 nmol/l. In those samples found to contain DDM the observed urinary concentrations were in the range 6 nmol DDM/mmol creatinine to 175 nmol DDM/mmol creatinine with a mean of 26 nmol DDM/mmol creatinine. All the factories used DDM in a solution with other components so that exposure by inhalation of dust or vapour was minimal. Skin absorption was considered the most probable source of exposure. All workers used protective clothing and gloves when handling DDM. The results from the five factories monitored in this study are summarised in table 1. On the days on which the

Fig 3 Frequency distribution of 323 samples submitted for routine analysis.

Table 1 Summary of urine results from five factories using DDM

Factory	No of samples (< 5 nmol DDM/mmol creatinine)	No of samples (> 5 nmol DDM/mmol creatinine)	Range
A	85	11	6-11
B	10	0	
C	111	11	6-42
D	20	47	6-175
E	24	5	6-17

Table 2 Percentage release of DDM

Time (min)	Mild hydrolysis			Strong hydrolysis		
	Exposed urine	Control + NADDM	DADDM	Exposed urine	Control + NADDM	DADDM
0	17	ND	ND	47	12	ND
15	43	ND	ND	52	74	68
30	53	ND	ND	68	97	98
60	47	ND	ND	100	100	100
90	45	ND	ND	100		
120	49	ND	ND	98		
150	48	ND	ND	100		

NADDM = N-acetyl DDM.
DADDM = N,N'-diacetyl DDM.
ND = None detected.

Fig 4 Electron impact mass spectra obtained after fractionation of urine from a worker exposed to (a) DDM and (b) pure N-acetyl DDM.

monitoring exercises were carried out, factory D was found to have the greatest evidence of absorption of DDM among the workforce whereas no DDM was found in the urine of the workers in factory B.

The results of the time course experiment in which DDM is released by two different hydrolysis procedures are shown in table 2. Under conditions of mild hydrolysis the concentration of ether extractable DDM increases with time. After about 30 minutes a plateau is reached with no further increase in DDM. Under conditions of strong (5M NaOH) hydrolysis, however, the concentration of DDM increases beyond that seen using mild hydrolysis and reaches a plateau after 60 minutes. The urinary DDM concentration produced by mild hydrolysis is approximately 48% of that seen after strong hydrolysis. The increase in DDM concentration under mild hydrolysis implies a thermally labile metabolite or conjugate, possibly an N-glucuronide. Using synthetic solutions of N-acetyl and N,N'-diacetyl DDM it has been shown that the mild hydrolysis procedure is ineffective in liberating DDM; however, the strong hydrolysis procedure will hydrolyse these compounds and release DDM. This response to mild and strong alkaline hydrolysis implied that the conjugate which hydrolysed under the more severe conditions might be an N-acetyl (mono or di) DDM. After HPLC, off-line mass spectrometry was used to look for acetyl DDM in the urine of three workers exposed to DDM. The spectra in figs 4a and 4b were obtained from a HPLC fraction of a worker's urine and synthetic N-acetyl DDM respectively. The spectra show close agreement and confirm the presence of acetyl DDM. The chromatographic fraction containing N-acetyl DDM had the same elution volume as synthetic N-acetyl DDM.

Hydrolysis of HPLC fractions, followed by analysis for DDM, showed DDM containing peaks with the same elution volume as DDM and N-acetyl DDM but not N,N'-diacetyl DDM. Therefore, if the increase in DDM concentrations seen between mild and strong hydrolysis is due to N-acetyl DDM, it appears that N-acetyl DDM is a major urinary metabolite of DDM.

Discussion

Until the metabolism of DDM has been fully characterised and the uptake and elimination kinetics have been determined, the method reported here for measuring "total" DDM offers a practical means of assessing occupational exposure. The concentration of DDM in urine may be used to reflect overall exposure and the extent of hygiene control in the factory. The control of exposure to aromatic amines relies on an understanding that minimising skin absorption and ingestion is as important as the

control of atmospheric contamination. The usefulness of environmental monitoring for assessing occupational exposure to industrial chemicals, which may enter the body by routes other than inhalation, is limited. In the case of potential carcinogens it is prudent to minimise exposure from all routes. Despite the fact that good hygiene is enforced and working practices are exemplary, absorption may occur.

MBOCA is an aromatic amine that is structurally similar to DDM. Recently, we have shown that biological monitoring can play an important part in a programme designed to reduce absorption of MBOCA. In a factory that manufactures polyurethane elastomers a monitoring programme over a five year period was used to provide evidence of absorption. We observed that improvements in the handling and use coincided with a steady reduction in urinary MBOCA concentrations.¹² A systematic biological monitoring programme will provide evidence of absorption of DDM and this may be used to initiate hygiene investigations to identify the source of exposure.

Here, we provide evidence for the existence of urinary metabolites of DDM. One of these metabolites has been identified as N-acetyl DDM and we suggest also that the N-glucuronides of DDM and N-acetyl DDM may exist. Lynn *et al* describe the occurrence of N-glucuronides of benzidine and acetyl benzidine.¹⁰ Both these conjugates are unstable in aqueous solution at neutral pH and room temperature, and their behaviour on hydrolysis appears to be similar to that of the labile conjugate of DDM. N-acetyl transferase is known to be involved in acetylation and thus in the deactivation of some arylamines which are potent carcinogens. This enzyme exhibits a polymorphism and has been suggested that slow acetylation affects the susceptibility to aromatic amine carcinogenesis. Cartwright and coworkers found an excess of slow acetylators among patients with bladder cancer who had been exposed to N-substituted aryl compounds.¹¹ We have provided evidence that N-acetyl DDM is a major urinary metabolite of DDM in man. By contrast, the chemically similar metabolite of MBOCA, N-acetyl MBOCA, is only a minor urinary metabolite.¹⁸ Glowinski *et al* has shown that the human polymorphic N-acetyl transferases are capable of N-acetylating MBOCA.¹⁹ The differences in urinary levels of acetyl metabolites may be due to differing affinities of the enzyme for the amine substrates. The toxicological significance of these metabolites depends on their subsequent metabolism. Recent work by Tanaka *et al*²⁰ and Hesbert *et al*²¹ shows that the N-acetyl derivatives of both DDM and MBOCA are less mutagenic than the parent amines in the Ames test. It is speculated that the route of activation of both amines to active mutagenic species is through

N-hydroxylation. The N-acetyl metabolite of DDM seen in human urine may therefore represent a detoxification mechanism. This may have implications for the slow acetylator phenotype in the exposed human population.

Conclusions

A new biological monitoring method has been developed for assessing occupational exposure to DDM. It is based on the quantitative determination of total (free and conjugated) DDM in human urine. A major metabolite of DDM has been isolated from human urine and has been identified as N-acetyl DDM. The strong hydrolysis procedure releases DDM from all the known metabolites and avoids the possible problems caused by hydrolysis of labile conjugates during sample transport to the laboratory and of variations caused by acetylator phenotype. The method, which uses GCMS, is sensitive and specific and is being used in routine monitoring exercises.

We are grateful to Dr D Gompertz for his encouragement and to Dr J D Thomas for his help and support during this study.

References

- 1 National Institute for Occupational Safety and Health Report. Background information on 4,4'-diaminodiphenylmethane (DDM). *Am Ind Hyg Assoc J* 1976;**37**:26-7.
- 2 Pludro G, Karlowski K, Mankowska M, Woggon H, Udhe WJ. Toxicology and chemical studies of some epoxy resins and hardeners. I. Determination of acute and sub-acute toxicity of phthalic acid anhydride, 4,4'-diaminodiphenylmethane and of the epoxy resin: Epilox EG-34. *Acta Pol Pharm* 1969;**26**:353-8.
- 3 Golke R, Schmidt P. 4,4'-Diaminodiphenylmethane: histological, enzyme histochemical and autoradiographic investigations in acute and sub-acute experiments in rats with and without the additional stress of heat. *Internationale Archiv für Arbeitsmedizin* 1974;**32**:217-31.
- 4 Deichmann WB, MacDonald WE, Coplan M, Woods F, Blum E. Di-(4-Aminophenyl)-methane (DDM): 4-7 year dog feeding study. *Toxicology* 1978;**11**:185-8.
- 5 Kopelman H, Robertson MH, Sanders PG, Ash I. The Epping jaundice. *Br Med J* 1966;**ii**:514-6.
- 6 Kopelman H, Scheur PJ, Williams R. The liver lesion of the Epping jaundice. *Q J Med* 1966;**35**:553-64.
- 7 Williams N, Sankey V, Bryan JA, Wolf FS. Toxic hepatitis and methylenedianiline. *N Engl J Med* 1974;**291**:1256-7.
- 8 McGill DB, Motto JD. An industrial outbreak of toxic hepatitis due to methylenedianiline. *N Engl J Med* 1974;**291**:278-82.
- 9 Ho T, Hardigree AA, Larimer FW, *et al.* Comparative mutagenicity study of potentially carcinogenic industrial compounds. *Environ Mutagen* 1979;**1**:167-71.
- 10 Steinhoff D, Grundmann E. Zur cancerogenen wirkung von 4,4'-diaminodiphenylmethane und 2,4'-diaminodiphenylmethane. *Naturwissenschaften* 1970;**57**:247-8.
- 11 Huff JE. Carcinogenesis results on seven amines, two phenols, and one diisocyanate used in plastics and synthetic elastomers. *Prog Clin Biol Res* 1983;**141**:347-63.
- 12 Thomas JD, Wilson HK. Biological monitoring of workers exposed to 4,4'-Methylene-bis-(2-chloroaniline) (MBOCA). *Br J Ind Med* 1984;**41**:547-51.
- 13 Nieminen EH, Saarinen LH, Laakso JT. Simultaneous determination of aromatic isocyanates and some carcinogenic amines in the work atmosphere by reversed-phase high-performance liquid chromatography. *Journal of Liquid Chromatography* 1983;**6**:453-69.
- 14 Tortoreto M, Catalani P, Bianchi M, Blonda C, Pantarotto C, Paglialonga S. Determination of 4,4'-diamino-diphenylmethane in blood by gas-liquid chromatography with electron-capture detection. *Journal of Chromatography* 1983;**262**:367-72.
- 15 Gristwood W, Robertson SM, Wilson HK. The determination of 4,4'-methylene-bis-(2-chloroaniline) in urine by electron capture gas chromatography. *J Anal Toxicol* 1984;**8**:101-5.
- 16 Lynn RK, Garvic-Gould C, Milam DF, *et al.* Metabolism of the human carcinogen, benzidine in the isolated perfused rat liver. *Drug Metab Dispos* 1983;**11**:109-14.
- 17 Cartwright RA, Glashan RW, Rogers HJ, *et al.* Role of N-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer. *Lancet* 1982;**ii**:842-6.
- 18 Ducos P, Marie C, Gaudin R. Assessment of occupational exposure to 4,4'-methylene bis (2-chloroaniline) "MOCA" by a new sensitive method for biological monitoring. *Int Arch Occup Environ Health* 1985;**55**:159-67.
- 19 Glowinski IB, Radtke HE, Weber WW. Genetic variation in N-acetylation of carcinogenic aryl amines by human and rabbit liver. *Mol Pharmacol* 1978;**14**:940-9.
- 20 Tanaka K, Ino T, Sawahata T, Marui S, Igaki H, Yashima H. Mutagenicity of N-acetyl and N,N'-diacetyl derivatives of 3 aromatic amines used as epoxy resin hardeners. *Mutat Res* 1985;**143**:11-5.
- 21 Hesbert A, Bottin MC, De Ceaurriz J. Mutagenicity of 4,4'-methylene-bis-(2-chloroaniline) "MOCA" and its N-acetyl derivatives in S typhimurium. *Int Arch Occup Environ Health* 1985;**55**:169-74.