Evidence that a β -N-glucuronide of 4,4'-methylenebis (2-chloroaniline) (MbOCA) is a major urinary metabolite in man: implications for biological monitoring

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Abstract

Urine samples from workers exposed to 4,4'-methylenebis (2-chloroaniline) (MbOCA) contain a labile metabolite(s) that, on hydrolysis, yields the parent compound at concentrations two to three times those of free MbOCA. Evidence has now been obtained that the major labile metabolite is an N-glucuronide of MbOCA. The N-glucuronide of MbOCA was synthesised chemically, characterised by thermospray mass spectrometry, and found to have a pseudomolecular ($M + 1$) ion at m/z 443/445. MbOCA and [^{14}C] uridine diphosphoglucuronic acid ([^{14}C]UDPGA) were incubated with liver microsomes from rats induced with polychlorinated biphenyls. The stoichiometry of the reaction product was about 1:1 (MbOCA:UDPGA). This product, the chemically synthesised glucuronide, and the labile urinary metabolite had identical chromatographic and hydrolytic (heat and β -glucuronidase) properties. These studies show that the major labile conjugate of MbOCA in the urine of workers exposed to this compound is probably the mono N-glucuronide. In view of the lability of this compound and the fact that its concentration in urine is two to three times that of free MbOCA, it is essential that any strategy for the biological monitoring of exposed workers takes into account the N-glucuronide.

The aromatic amine 4,4'-methylenebis (2-chloroaniline) (MbOCA) is used in the plastics industry to cure urethane elastomers, epoxyresins, and isocyanates. It has attracted the attention of occupational

physicians and the regulatory authorities after reports that it is a carcinogen in at least three mammalian species.¹⁻³ Experimental studies have shown that MbOCA is mutagenic with both rat and human hepatic S9⁴ and may form adducts with DNA in the rat⁵ and in human bladder explants.⁶ MbOCA has also been implicated in two cases of bladder tumours in young men who were occupationally exposed to the compound.⁷

Occupational exposure to aromatic amines may be assessed by a programme including biological monitoring rather than by environmental monitoring alone. This is particularly important as skin absorption may be a major route of entry into the body. In the case of MbOCA it has been shown that biological monitoring coupled with the complementary approach of occupational hygiene investigation can significantly reduce exposure.⁸ With the early analytical methods only "free" MbOCA was measured in urine. In 1984, however, this laboratory reported the presence of a labile MbOCA conjugate(s) in the urine of exposed workers.⁹ This conjugate(s) was first suspected when it was observed that the concentration of MbOCA in urine increased two to fivefold from when the free amine was measured immediately after sample collection to reanalysis 24 hours after being left at room temperature. Mild hydrolysis of the urine samples, by heat at neutral pH, resulted in similar increases in the concentration of free MbOCA.

Such ease of hydrolysis is characteristic of the N-glucuronides of several aromatic amines, including benzidine, naphthylamine, and aminobiphenyl.¹⁰⁻¹³ In an attempt to confirm the identity of this conjugate and to assess its possible implications for the biological monitoring of MbOCA we have compared the chromatographic behaviour, enzymatic hydrolysis, and mass spectra of synthetic N-glucuronides of MbOCA with the labile conjugate(s) found in the urine of workers occupationally exposed to the amine.

Materials and methods

URINE SAMPLES

Spot urine samples were collected in universal con-

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tainers at the end of an eight hour working shift from workers occupationally exposed to MbOCA as part of a routine programme of biological monitoring. The samples were frozen immediately and transported on cardice to the laboratory where they were stored at -20°C until analysis.

SYNTHESIS OF MBOCA-N-GLUCURONIDES

The chemical synthesis of the N-glucuronide of MbOCA was based on a modification¹¹ of the method of Boyland *et al.*¹³ MbOCA (10 mM in ethanol) was mixed with an equal volume of glucuronic acid sodium salt (20 mM in sodium phosphate buffer 0.1 M, pH 7.1) and the mixture left in the dark at $0-4^{\circ}\text{C}$ for 16 hours.

The enzymatic synthesis of the N-[^{14}C]glucuronide of MbOCA, by hepatic microsomal fraction from polychlorinated biphenyl (PCB) induced rats, was also based on the method of Lilienblum and Bock,¹¹ modified to include the use of [^{14}C]uridine diphosphoglucuronic acid ([^{14}C]UDPGA). All reagents, except UDPGA and [^{14}C]UDPGA, were added, mixed, and preincubated at 37°C for two minutes before the addition of UDPGA. Incubation mixtures contained Tris HCl buffer (75 mM), MgCl_2 (5 mM), Brij 58 (0.5 mg/ml), amine solution (0.5 mM in ethanol), microsomal protein (1 mg), and [^{14}C] UDPGA (3 mM, specific activity 5.2 mCi/mmol) and were maintained at 37°C for 15 minutes.

SEPARATION AND CHARACTERISATION OF N-GLUCURONIDES

High performance liquid chromatography (HPLC) purification of the N-glucuronides of MbOCA was achieved using a Nova Pak 5 μm column 10 cm $\times$ 8 mm (Waters Radial Pak) with gradient elution (Waters model 510). The mobile phase comprised a mixture of methanol and water containing PICA (tetrabutylammonium phosphate) (flow rate, 2 ml/min). The composition of the mobile phase was held at 30% methanol: 70% aqueous PICA for two minutes then increased to 100% methanol over the next 25 minutes.

ASSAY OF MBOCA IN HPLC FRACTIONS

HPLC method for the assay of MbOCA

After HPLC separation as described above, eluate fractions (1 ml) were collected. These were mixed with dichlorobenzidine as internal standard (1 ml, 1 $\mu\text{mol/l}$). After hydrolysis (see below), the samples were extracted with diethyl ether (7 ml). The ethereal layer was removed and evaporated to dryness at 40°C in a vortex evaporator. The residue was dissolved in a mobile phase (1 ml). Fifty microlitre samples were injected on to a 5 μm Radial Pak C18 column with a mobile phase of 50% acetonitrile: 50% NaH_2PO_4 buffer (50 mM, pH 7.4) at a flow rate of 2 ml/min, using an automated injection system.

The eluate was monitored with an electrochemical detector (Coulchem) with the polarising voltage set at +1 V. MbOCA standards were prepared in water and subjected to the same hydrolysis and extraction procedures as the urine fractions.

GCMS method for the assay of MbOCA

MbOCA was measured by a modification of our previous method.¹⁴ Internal standard (4,4'-methylene dianiline (MDA)) (1 ml, 1 $\mu\text{mol/l}$) was added to the HPL eluate fractions, which were then hydrolysed by heating to 80°C for one hour. Aliquots (1 ml) were made alkaline with 10 M NaOH (100 μl) and extracted with diethyl ether (7 ml). After evaporation of the ether, the residues were derivatised with heptafluorobutyryl chloride (1 ml, 1% v/v in cyclohexane) at 80°C for one hour. Excess derivatising reagent was removed by evaporation and the residues dissolved in toluene (100 μl). Aliquots (1 μl) were injected into the GCMS, operating in splitless mode (30 sec) at an oven temperature of 200°C , which was then increased at 30°C a minute to 300°C .

Mass spectrometer conditions were as follows: electron impact ionisation (70 eV, 200 μA), source temperature 240°C , selected ion monitoring using voltage switching for ions m/z 590 (MDA), and m/z 623 (MbOCA), both with a dwelling time of 100 msec. Standards were prepared by adding MbOCA solution (1 $\mu\text{mol/l}$) to 1 ml aliquots of water and then processing as for the samples.

Enzymatic hydrolysis of MbOCA conjugates

The chemically synthesised N-glucuronides of MbOCA were fractionated by HPLC and then hydrolysed with β -glucuronidase. Aliquots of the incubation mixture (500 μl) were removed at timed intervals and the reaction stopped by the addition of saccharo-1,4-lactone (100 μl , 100 mM) containing internal standard. Hydrolysis was carried out at 37°C with β -glucuronidase (Sigma type VII, 1000 units) in phosphate buffer (14 mM, pH 6.8). The progress of hydrolysis was assessed from the amount of free MbOCA liberated.

Determination of the molar ratio of glucuronic acid to MbOCA in the glucuronide of MbOCA

The enzymatically synthesised glucuronide of MbOCA was subjected to HPLC and those fractions of eluate corresponding to the N-glucuronide peak were collected. The "glucuronide fractions" (2 ml) were each divided and half of each was used to determine the glucuronic acid content and the remainder for MbOCA analysis.

The amount of radioactivity in the HPLC fractions was determined by liquid scintillation spectrometry, using an LKB Rak Beta scintillation counter with quench correction by the external channels ratio method. The glucuronic acid content in each

fraction was calculated from the number of disintegrations per second (corrected for background) and the specific activity of the [^{14}C]UDPGA.

The amount of MbOCA in the HPLC fractions was determined after hydrolysis of the conjugate by the addition of hydrochloric acid (50 μl , 5 M) and heating to 90°C for one hour followed by addition of sodium hydroxide (100 μl , 10 M) and heating to 90°C for a further hour. Standard MbOCA (and internal standard) solutions (0–1 nmol tube) were prepared and subjected to the same hydrolysis procedure. MbOCA liberated by this procedure was analysed as described earlier.

Identification of MbOCA N-glucuronide by mass spectrometry

The HPLC fractionated conjugates of MbOCA (5 μl) were subjected to direct probe mass spectrometry using a VG16F mass spectrometer and data system (VG Analytical), using both electron impact (70 eV) and chemical ionisation modes. In view of the labile nature of the conjugates, samples were frozen on to the tip of the probe by immersing it in liquid nitrogen. The probe was then immediately inserted into the mass spectrometer.

Both EI and CI mass spectra showed ions characteristic of the parent compound, MbOCA, and some low intensity ions at a higher m/z than that of the molecular ion of MbOCA. These, however, could not be assigned as fragment ions of the molecular ions of the N-glucuronides.

Thermospray mass spectra were obtained using a Finnigan 4500 mass spectrometer. Chemically synthesised N-glucuronides of MbOCA (50 μl of reaction mixture) were injected on to a Nova Pak C18 column; the mobile phase was ammonium acetate (50 mM): acetonitrile at a flow rate of 1.9 ml/min. The acetonitrile content was increased from 0 to 50% over 15 minutes. The source temperature was 180°C and the vaporiser temperature 130°C.

Analysis of urine from workers exposed to MbOCA

Samples of urine (1 ml) from workers exposed to MbOCA were injected into the HPLC using conditions similar to those described earlier for the purification of the N-glucuronide conjugates. The urine samples were injected immediately after thawing or after heating at 80°C for 45 minutes. Fractions eluting from the HPLC were analysed for MbOCA content by either GCMS or HPLC, using the methods described above.

Results

CHARACTERISATION OF MBOCA-N-GLUCURONIDES

HPLC separation of the products from the chemical synthesis of the N-glucuronides of MbOCA gave two ultraviolet absorbing peaks which eluted earlier than free MbOCA (fig 1). When the HPLC eluate corre-

sponding to these two peaks was collected and reinjected on to the HPLC column, a peak corresponding to free MbOCA appeared, with a parallel decrease in the original conjugate peak. Thus these peaks both contained labile derivatives of MbOCA that were more polar than MbOCA itself and which were readily hydrolysed back to the parent amine. The likely identities of the two peaks were the α - and β -N-glucuronides of MbOCA. Nevertheless, attempts to obtain EI and CI mass spectra and molecular weight information for these peaks failed. The absence of molecular ions in the mass spectra was probably due to the chemical instability of the conjugates, with decomposition occurring before or during introduction into the mass spectrometer.

The β -N-glucuronide of MbOCA was specifically synthesised by incubation of the amine with UDPGA and rat hepatic microsomes. By using radiolabelled UDPGA it would be possible to determine the molar ratio of glucuronic acid to the amine in the conjugate. In contrast to the chemical synthesis, the enzymatic synthesis produced only one HPLC peak in addition to MbOCA, which had the

Figure 1 HPLC chromatogram of mixture obtained from chemical synthesis of N-glucuronides of MbOCA: 1 β -N-glucuronide, 2 α -N-glucuronide, and 3 parent amine.

Figure 2 Reconstituted ion chromatogram from HPLC-MS analysis of MbOCA-N-glucuronide synthetic mixture. Peaks 1 and 2 = β - and α -N-glucuronides of MbOCA respectively, peak 3 = MbOCA.

same retention time as the earlier (more polar) of the two conjugate peaks synthesised chemically. The formation of only one conjugate peak was as expected, given the isomeric specificity of microsomal uridine 5'-diphosphoglucuronyl transferases, which produce glucuronides only in the β configuration.

The MbOCA content of this peak was determined by GC-MS and the glucuronic acid content by liquid scintillation spectrometry. The mean ($n = 3$) molar ratio of glucuronic acid to MbOCA in this conjugate was 1:1.24 and 0.9:1 in two separate experiments. The molar ratio is thus close to 1:1, showing that the conjugate was a monoglucuronide of MbOCA.

The structure of the glucuronide conjugate was investigated further by HPLC thermospray mass spectrometry. The reconstituted ion chromatogram obtained on HPLC-MS analysis of the chemically synthesised MbOCA-glucuronide mixture (fig 2) was similar in appearance to the HPLC chromatogram for the mixture, shown in fig 1. Figure 3 shows the mass spectrum of the first, more polar adduct of MbOCA, the putative β -isomer. The spectrum is dominated by ions at m/z 267–269 but also shows a prominent ions at m/z 443–445, the relative intensities of which suggest the presence of two chlorine atoms. The m/z values of these latter

ions are consistent with those of the protonated molecular ions of an N-glucuronide conjugate of MbOCA. Whereas an ion was observed at m/z 619 ($M + 1$ of the N,N'-diglucuronide of MbOCA) its intensity was <0.5% of that of the base peak and it did not contain two chlorine atoms. Thus this ion is unlikely to derive from an N,N'-diglucuronide of MbOCA. The ions at m/z 267–269 may be assigned as the protonated molecular ions of MbOCA (the molecular weight of MbOCA is 266–268), formed by partial hydrolysis of the N-glucuronide conjugate in the thermospray interface (at 135°C), giving rise to substantial amounts of free MbOCA. This interpretation is supported by the absence of fragmentation from the pseudomolecular ion of MbOCA (at m/z 267–269), indicating that under the mild thermospray ionisation conditions, MbOCA entered the mass spectrometer as the free amine (formed in the interface by hydrolysis), rather than as a fragment ion from the glucuronide conjugate. The second conjugate peak, the putative α -isomer, produced a mass spectrum almost identical to that of the β -isomer (data not shown).

The similarity of the mass spectra of two chromatographically separable peaks strongly suggests that the compounds were isomers, most likely

Figure 3 Thermospray mass spectrum of β -N-glucuronide adduct of MbOCA.

the β - and α -N-glucuronides of MbOCA, respectively.

Figure 4 shows the effects of chemical and enzymatic hydrolysis of the α - and β -N-glucuronides of MbOCA. The more polar (larger) of the HPLC peaks was rapidly hydrolysed by β -glucuronidase. The β -glucuronidase inhibitor saccharo-1,4-lactone reduced hydrolysis to a rate similar to that occurring spontaneously on incubation at 37°C in the absence

of enzyme. These results provide further evidence for the identity of this conjugate as the β -glucuronide of MbOCA.

The second, less polar, of the HPLC peaks was not readily hydrolysed by β -glucuronidase, the rate of hydrolysis in the presence of enzyme being similar to that at 37°C in the absence of enzyme (fig 4). Saccharo-1,4-lactone had no effect on the hydrolysis of this conjugate in the presence of β -glucuronidase.

Figure 4 Hydrolysis of α - and β -N-glucuronides of MbOCA by heat at 37°C (Δ), heat at 80°C ($\blacktriangle$), β -glucuronidase ($\bullet$), and β -glucuronidase + saccharo 1,4-lactone ($\circ$). Results have been expressed as percentage hydrolysis and are means of duplicate determinations.

Figure 5 Partial HPLC-UV 254 chromatograms obtained from injections of 200 μ l of (a) synthetic N-glucuronide mixture, (b) urine from a worker not exposed to MbOCA, (c) urine from a worker exposed to MbOCA, and (d) concentration of MbOCA in fractions of eluate collected from (c) and determined by GCMS after alkaline hydrolysis. Fractions (1 ml) were collected at 30 second intervals from time of injection. Peak 1 = β -N-glucuronide of MbOCA, peak 2 = MbOCA.

Figure 6 Histogram of concentration of MbOCA in HPLC fractions (after alkaline hydrolysis) of urine samples obtained from worker exposed to MbOCA. Peak identifications, based on HPLC retention time, are: 1—MbOCA β -N-glucuronide, 2—diacetyl MbOCA + unknown, 3—N-acetyl MbOCA, 4—MbOCA. Fractions (1 ml) were collected at 30 second intervals from time of injection. Each point is mean of duplicate determinations performed in separate experiments.

These data are consistent with the assignment of this conjugate as the α -N-glucuronide of MbOCA.

Both conjugates underwent non-enzymatic hydrolysis, the α -glucuronide showing somewhat greater lability than the β -isomer. At 37°C, over 60% of the β -glucuronide was hydrolysed within three hours and 70% of the α -glucuronide within one hour. Hydrolysis of both conjugates was increased with temperature and was complete within 90 minutes at 80°C.

IDENTIFICATION OF N- β -GLUCURONIDE OF MBOCA IN URINE FROM OCCUPATIONALLY EXPOSED WORKERS

Urine samples from workers potentially exposed to MbOCA were analysed for glucuronides of the amine in the same way as was used for the chemically synthesised conjugates. Samples were fractionated by HPLC and the fractions hydrolysed and analysed quantitatively for MbOCA. Some urine samples were concentrated fivefold by freeze drying and then reconstituted in 0.2 vol of HPLC mobile phase. On HPLC, such samples showed an ultra violet absorbing peak with the same retention time as that of the β -N-glucuronide of MbOCA, which was not present in urine samples from subjects not exposed to MbOCA (fig 5). The ultraviolet peak in the urine samples from exposed workers yielded MbOCA on hydrolysis.

Urine samples from workers exposed to MbOCA were fractionated by HPLC and the hydrolysed fractions analysed for MbOCA by HPLC with electrochemical detection. At least four different MbOCA containing peaks were found (fig 6). The first of these peaks had the same retention time as that of the MbOCA β -N-glucuronide and disappeared

after heat hydrolysis of the urine sample before HPLC fractionation (fig 6). Although the second peak had the same retention time as that of diacetyl MbOCA, it, too, disappeared after initial heat hydrolysis, showing that this was not diacetyl MbOCA but some other minor labile conjugate. The third peak had the same retention time as that of monoacetyl MbOCA and changed little on heat pretreatment of the urine samples. The fourth peak had the same retention time as that of free MbOCA, and increased considerably after heat hydrolysis of the urine samples due to the liberation of free amine on hydrolysis of its N-glucuronide. Quantitatively, the most significant MbOCA containing metabolite in the urine of two workers exposed to MbOCA was the β -N-glucuronide. After heat hydrolysis of the urine samples the major chromatographic peak was free MbOCA.

Discussion

Biological monitoring has been used to measure the absorption of MbOCA by individual workers and has helped in controlling occupational exposure. This has been achieved by the quantitative analysis of MbOCA in workers' urine. Thomas and Wilson observed that improvements in the handling and use of MbOCA coincided with a steady reduction in urinary MbOCA concentrations.⁸

Although there have been considerable improvements in the methods available for analysing urinary MbOCA, only two reports mention the presence of labile conjugates.^{8,14} N-Centres can form N-glucuronides, N-sulphamates, and N-carbamates, all of which are readily hydrolysed.¹⁵ N-Glucuronides, in particular, have been identified as conjugates of the aromatic amines, naphthylamine, benzidine, and 4-aminobiphenyl.¹⁰⁻¹³ The synthetic N-glucuronide of MbOCA reported in this paper has characteristics similar to those of the N-glucuronide of benzidine.¹⁰ Both MbOCA and benzidine glucuronides spontaneously decompose in aqueous solution at 37°C within 24 hours to yield the parent amines. In addition, neither glucuronide produces a molecular ion on electron impact mass spectrometry, the only major ions produced being characteristic of the respective free amines.

In the present study chemical synthesis resulted in the formation of two N-glucuronides of MbOCA. The more polar of these was readily hydrolysed by β -glucuronidase, had a molar ratio of glucuronic acid to MbOCA of about 1:1, and had a thermospray mass spectrum consistent with that of a mono-N-glucuronide. The chromatographic properties of this conjugate on HPLC were identical to those of the enzymatically synthesised β -N-glucuronide. Thus it is concluded that this compound was the β -N-glucuronide of MbOCA and that the other product of chemical synthesis was the α -N-glucuronide.

Urine from workers occupationally exposed to MbOCA contains a labile metabolite that has the same retention time on HPLC as the β -N-glucuronide of MbOCA and that yields MbOCA on hydrolysis. Furthermore, if urine samples are heated before HPLC fractionation this labile metabolite disappears and there is a corresponding increase in the concentration of free MbOCA. Thus the most likely nature of the labile metabolite of MbOCA in workers' urine is the β -N-glucuronide of the amine.

Although the β -glucuronide is the major MbOCA containing metabolite in the urine of workers exposed to the compound, at least three other MbOCA related compounds are present. One of these is free MbOCA. Another resembles N-acetyl-MbOCA in its retention time and resistance to heat hydrolysis. The remaining compound is heat labile, and may be a conjugate of N-acetyl-MbOCA.

MbOCA β -N-glucuronide is readily hydrolysed both spontaneously at 37°C and by β -glucuronidase. Urine normally contains β -glucuronidase but this varies in concentration over a 10-fold range.¹⁶ It is possible that little or no MbOCA is excreted into the bladder as the free amine, with most of the MbOCA present as the N-glucuronide. This is likely to undergo some hydrolysis in the bladder, either enzymatically or spontaneously, before voiding of the urine. Furthermore, urine samples from workers in factories sent by post to the laboratory for analysis are likely to be subjected to considerable variation in ambient temperature and time between voiding and analysis. Measurement of unconjugated ("free") MbOCA in these samples will therefore be greatly influenced by the preanalytical treatment of the sample, leading to erroneous estimates of relative exposure. Reliable determination of exposure will thus be obtained only after complete hydrolysis of any labile conjugates. Any biological monitoring strategy for workers who might be exposed to MbOCA should include measurement of "total" urinary MbOCA after hydrolysis.

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