Methylene bis (2-chloroaniline) (MbOCA): towards a biological monitoring guidance value

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We report the development and validation of a high performance liquid chromatography method for the determination of methylene bis (2-chloroaniline) (MbOCA) and its labile conjugates in urine. The method has been in regular use for the biological monitoring of workers exposed to MbOCA for the past 11 years. Following the development of a biological monitoring strategy, and the introduction of a biological action level by the Health and Safety Commission in 1984, there has been a steady fall in the proportion of workers whose urinary results are above the action level. We conclude that, in the absence of reliable health-based data, a guidance value based on the use of the 90th percentile derived from monitoring a cross-section of the industry, can be used to interpret biological monitoring results. The measurement of urinary MbOCA is a practical non-invasive way of monitoring workers which can be useful in helping to control exposure.

Keywords: *biological monitoring, methylene bis(2-chloroaniline), MbOCA, high performance liquid chromatography, guidance value.*

Introduction

Methylene bis (2-chloroaniline) (MbOCA) is used in the manufacture of some polyurethanes and elastomers which have a range of uses from wear-resistant linings for pulley wheels and metal containers to seals and gaskets. MbOCA in its pure form is a solid at room temperature with low vapour pressure and unless it is used in a way which generates dusts, the major route of occupational exposure is via the skin rather than inhalation (Smith and Woodward 1983, Thomas and Wilson 1984, Hotchkiss et al. 1993). There is concern about occupational exposure to MbOCA since it is genotoxic *in vitro* and carcinogenic in at least two animal species and there is some suggestion that it may be a human bladder carcinogen (Ward et al. 1987, McQueen and Williams 1990). MbOCA is currently classified in the EC as a category 2 carcinogen and is defined as a carcinogen under the Control of Substances Hazardous to Health (COSHH) by virtue of its R45 classification.

In recent years the use of biomarkers has developed to the stage where they are seen as useful tools for monitoring occupational exposure and for assisting in making health risk assessments. Since biological monitoring data give an estimate of uptake by all exposure routes (inhalation, ingestion and skin absorption) the measurement is related to body burden and hence to individual health risk, albeit with a dose–response

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relationship which is not well defined. The usefulness of biological monitoring is increased if the results can be interpreted according to appropriately set guidance values. Ideally, these levels should be health-based and derived from dose response data. However, MbOCA is a potential human carcinogen and a health-based limit or a no-effect level cannot be identified. An alternative approach adopted by the Health and Safety Executive, is to set a value based on exposure data or what is observed to be good hygiene practice throughout the industry. If sufficient data from a cross-section of the industry are available then it is possible to set levels or 'benchmark' values based on what can be achieved through good practice. Such an approach was proposed for the chemically similar, methylene dianiline (Cocker et al. 1994). The rationale for setting a benchmark is to give an indication when control measures need to be examined. It should therefore spur improvements in control procedures within the factory which should then have the effect of reducing exposure to potentially toxic substances.

The most widely used procedure for monitoring MbOCA exposure is the measurement of MbOCA in urine (Linch et al. 1971, Thomas and Wilson 1984, Ducos et al. 1985, Lowry and Clapp 1992). The only human urinary metabolites of MbOCA found so far are MbOCA *per se*, the *N*-acetyl and *N,N'*-diacetyl and *N*-glucuronide conjugates (Cocker et al. 1988, 1990). The acetyl conjugates of MbOCA are minor metabolites, whereas the *N*-glucuronide is typically present in amounts two to three times that of unchanged MbOCA. We have argued that the measurement of 'total' urinary MbOCA should be used for monitoring purposes. By 'total' we mean, MbOCA which is excreted unchanged and that which is readily released from labile conjugates (Cocker and Wilson 1989). More recently two other methods have been reported. They may have some applications in monitoring worker exposure and possibly biological effect but so far these methods, MbOCA-DNA adducts in exfoliated urothelial cells (Kaderlick et al. 1993) and MbOCA-haemoglobin adducts (Bailey et al. 1993) have not been taken into routine use.

In 1977 a biological monitoring programme for workers exposed to MbOCA was made available in the United Kingdom by the Health and Safety Executive (HSE). The programme was based on the measurement of 'free' MbOCA in urine (Thomas and Wilson 1984). Subsequently an improved analytical method was developed to measure both free and heat-labile conjugates ('total') MbOCA (Gristwood et al. 1984). Both methods were used in parallel for the following four years after which the 'total' MbOCA method was taken into regular use. In July 1984 the Health and Safety Commission (HSC) set an advisory biological action level of 10 nmol 'free' MbOCA per mmol creatinine. Industry was advised that where biological monitoring results showed levels of MbOCA in urine higher than the action level, employers should carry out a hygiene survey to find out the source of MbOCA exposure and initiate improved control measures. In July 1987 a new biological action level for 'total' MbOCA was set at 30 nmol mmol^{-1} creatinine (HSC 1987) to allow the improved analytical method to be used (10 nmol 'free' MbOCA is approximately equivalent to 30 nmol 'total' MbOCA).

In this paper we report in detail the high performance liquid chromatographic (HPLC) method used in the Health and Safety Laboratory for measuring 'total' MbOCA in urine. We report in summary form, the MbOCA results generated by this laboratory from monitoring UK industry from 1977 to 1994, and present an analysis of the data collected over the past 11 years. In particular, we set out to examine whether the introduction of an advisory biological action limit was effective in helping to reduce exposure to MbOCA.

METHODS

Principle of the method

The analysis of MbOCA in urine is based on the hydrolysis of labile conjugates of MbOCA by heat, followed by extraction from the urine and HPLC analysis with electrochemical detection.

Collection of urine samples

Although HSE field operations staff encouraged MbOCA firms to participate in the biological monitoring exercise, the scheme was voluntary. Since the exercise began in 1977 we have received samples from over 50 firms. The majority of urine samples, sent in routinely to our laboratory, came from workers in a core of 25 factories which used MbOCA. Urine samples generally were collected at the end of a working shift in 30 ml plastic containers. Samples were delivered to the laboratory within 48 hours and stored at -20 °C until ready for analysis. MbOCA in urine samples stored this way were found to be stable for at least 3 months (Gristwood *et al.* 1984, Jedrzejczak and Gaind 1992).

Reagents

The MbOCA used was purchased from Ralph Emanuelle or Pfaltz and Bauer, Waterburg, USA and recrystallized from methanol. The 3,3' dichlorobenzidine (DCB—internal standard) came from Sigma, Poole, Dorset. Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, peroxide free diethyl ether, HPLC grade methanol, water, and acetonitrile were purchased from Fisons, Loughborough. (NB: MbOCA and DCB are potential carcinogens and precautions should be taken to avoid exposure, particularly when weighing out solid material.)

Equipment

A waterbath or dry-block was used for sample hydrolysis. A rotary tumbler/mixer, a centrifuge and solvent evaporation equipment was used for the solvent extraction method. Disposable extraction columns packed with 100 mg C_{18} stationary phase, and an extraction manifold were used for the solid-phase extraction technique (Isolute International Sorbant Technology, Hengoed, Mid Glamorgan). A Gibson Model 305 HPLC and manometric module was used with a Coulochem 5100A electrochemical detector. The system was fitted with a 125 mm × 4.6 mm column packed with a 3 μm Spherisorb ODS2 (octadecylsilane 12 % w/w carbon loading) from Phase Sep, Clwyd, UK.

Calibration

The purity of the standard MbOCA was verified by HPLC and GCMS. Standard solutions of MbOCA (1mM) and internal standard dichlorobenzidine (1 mM) were prepared in methanol, stored in the dark at 4 °C and discarded after 1 month. Daily working solutions of MbOCA (4 μM), and DCB (5 μM) were prepared by dilution in water. The MbOCA working solution was used to spike aliquots of urine (2 ml) from an unexposed individual to give a calibration curve from 0 to 500 nM MbOCA; standards were taken through the procedure below.

Sample preparation

Aliquots of urine (2 ml in duplicate) were transferred to screw-capped glass tubes, spiked with the internal standard DCB (100 μl of 5 μM), capped, mixed and heated at 100 °C for 30 min (Gristwood *et al.* 1984, Cocker *et al.* 1990). The samples were allowed to cool to room temperature and the MbOCA was extracted, either by solvent extraction or by solid phase extraction.

For *solvent extraction*, sodium hydroxide (1 ml, 1 M) and diethyl ether (8 ml) were added to each tube and the contents mixed for 30 min. The tubes were then centrifuged at 900 × *g* for 10 min to separate the aqueous and organic phases. The diethyl ether layer was transferred to a clean tube and the solvent evaporated to dryness at 40 °C under a gentle stream of oxygen-free nitrogen. Once dry the residues were reconstituted using HPLC mobile phase (1 ml) and subjected (200 μl) to HPLC analysis.

For *solid phase extraction*, the disposable extraction columns packed with 100 mg of C_{18} were each conditioned with 2 ml of methanol followed by 2 ml of water. Aliquots of hydrolysed samples (0.8 ml) were transferred to the extraction columns and washed with water (0.8 ml) and 45 % acetonitrile in water (0.8 ml). The MbOCA was then eluted into collection tubes with 90 % acetonitrile in water (0.3 ml) and diluted with phosphate buffer (0.4 ml, 50 mM, pH7) before injection (200 μl) into the HPLC system.

HPLC analysis

The extracts prepared either by solvent extraction or by solid phase extraction were analysed using a single HPLC pump with an isocratic mobile phase (phosphate buffer (50 mM, pH7): acetonitrile: methanol (12:10:1) at a flow rate of 1 ml min⁻¹ into an 125 mm × 4.6 mm 3 μm ODS column connected to an electrochemical detector with a polarizing voltage of + 0.7 V. Peak height ratios were calculated for the MbOCA and DCB peaks which eluted after about 7.5 and 6 mins respectively under the conditions described above. The concentration of MbOCA in the samples was calculated by comparison of the peak height ratio to the calibration curve determined by linear regression analysis of the calibrants prepared and analysed at the same time as the unknown urine samples.

Quality control (QC)

This laboratory operates a quality control scheme for urinary MbOCA based on urine spiked with MbOCA at about 270 nmol l⁻¹. The QC material was aliquoted into 5 ml vials and these were then freeze-dried, sealed and stored at -20 °C. QC samples were run before and after each batch of five samples.

Creatinine correction

Creatinine was measured in all urine samples by the method of Jaffe (1886) and the MbOCA results were expressed as μmol MbOCA per mol creatinine to compensate for concentration/dilution effects. Opinion is divided on the benefit of creatinine correction. Biological monitoring surveys for MbOCA include those which correct for creatinine (Will *et al.* 1981, Gristwood *et al.* 1984, Jedrzejczak and Gaind 1992) and those that do not (McKerrell *et al.* 1987).

Statistical analysis

The data were analysed based on the X^2 test for linear trend using a contingency table.

Results

Performance of the HPLC method

The HPLC method has been in routine operation in our laboratory for 11 years and has proved to be a reliable and robust technique. A typical HPLC trace is shown in Figure 1. The recovery for the solvent extraction method was 94 % and
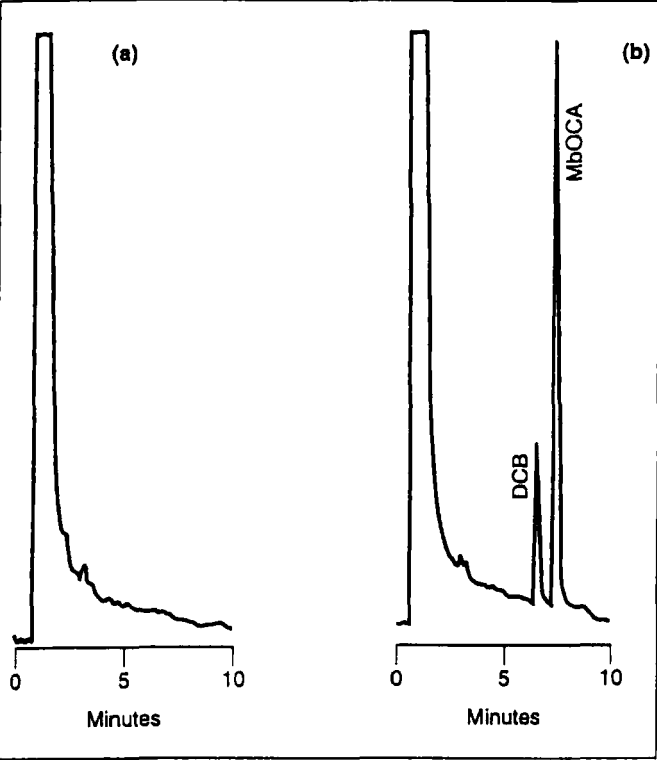
Figure 1. Typical HPLC trace of (a) blank and (b) urine from MbOCA-exposed worker with added DCB as internal standard.

Year	Number of urine samples	90th percentile (μmol MbOCA per mol creatinine)
1977	1017	180
1983	1280	30
1987	1142	20
1993/94	629	15

Table 1. 90th percentile of urinary MbOCA results for the years 1977 until 1994.

Year	Total number of samples	Number of factories
1983	1280	21
1984	1880	22
1985	1838	21
1986	1428	21
1987	1142	22
1988	832	21
1989	1166	22
1990	1092	23
1991	806	21
1992	752	22
1993	394	16
1994	235	12

Table 2. Number of samples analysed and number of factories monitored from 1983 until 1994.

greater than 95 % for the solid phase method. Comparison of the solvent extraction method with the solid phase extraction method showed good agreement between the methods (coefficient of determination of 0.9925 and a slope of 1.021). Comparison of the solvent extraction-HPLC procedure with a GCMS method showed a correlation coefficient of 0.976 and a slope of 1.075 in the range of 0.03–11 μM (Osorio *et al.* 1990).

The precision, expressed as relative standard deviation at 250 nmol l^{-1} was typically 3 % and 7 % for within-run and between run analyses respectively for the solvent extraction procedure. The within-run and day to day relative standard deviations for the solid phase procedure were typically 2 % and 5 % respectively. The limit of quantitation using this method, based on peaks greater than three times background noise and half the lowest point on the calibration curve was 25 nM.

Urinary MbOCA results from 1977 to 1994

In 1977 the 90th percentile of results from samples measured by our laboratory in terms of 'total' MbOCA was 180 μmol mol^{-1}. By 1994 the 90th percentile had fallen to 15 μmol mol^{-1} (Table 1). We have examined the urinary MbOCA data from 1983 until 1994. The number of factories monitored and the number of samples submitted is given in Table 2. Analysis of the data from all MbOCA factories monitored from March 1983 to September 1994 shows that there is a highly significant downward linear trend in the proportion of workers having urine values which exceed the action level ($p < 0.0001$) (Figure 2). There was also a corresponding significant upward linear trend ($p < 0.0001$) in
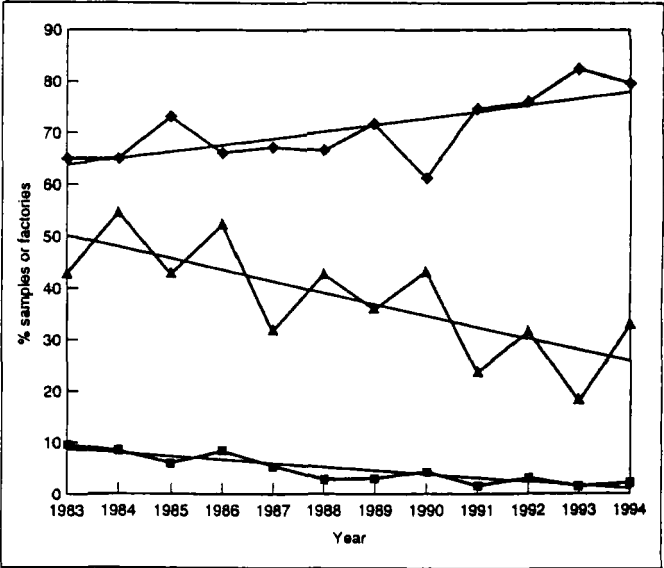

Figure 2. Trend of urinary MbOCA results from 1983 to 1994 plotted as % samples measured or factories monitored. ◆, % samples with urinary MbOCA levels < 4 μmol mol^{-1} ; ■, % samples with urinary MbOCA levels > 30 μmol mol^{-1}; ▲, % factories with workers who had urinary MbOCA levels > 30 μmol mol^{-1}; ——, trend analysis.

the proportion of workers with negligible urinary MbOCA levels (i.e. none detected to 4 μmol mol^{-1}. There was a less obvious but significant downward trend ($p = 0.079$) over the 11-year period in the number of firms who had workers with urine values which exceeded the action level. In 1983 10 % of

Year	Total number of samples	Number of factories	% samples in range (μmol mol^{-1})				
			None detected	1–4	5–15	16–30	>30
1987	1142	22	46.3	21.2	12.6	14.4	5.5
1988	832	21	48.6	18.1	17.3	13.1	2.9
1989	1166	22	49.4	22.5	9.1	16	3
1990	1092	23	41.9	19.6	24.6	9.8	4.3
1991	806	21	57.9	16.7	16	7.6	1.8
1992	752	22	59.2	17	11.6	9.2	3
1993	394	16	64.5	17.9	7.6	8.1	1.9
1994	235	12	65.5	15.1	12	5.3	2.1

Table 3. Percentage of urine samples collected between 1987 and 1994 with MbOCA concentrations falling within certain limits. The data have been taken from a core of 25 factories.

samples were above the action level and by 1987 it had fallen to 5.5 % with a further reduction by 1994 to 2 % (Figure 2).

Detailed analysis of the data from 1987 until 1994 gives the distribution of samples against the urinary MbOCA concentrations and shows that the current 90th percentile is about 15 μmol MbOCA per mol creatinine (Table 3).

Discussion

The high performance liquid chromatography method reported here for measuring unchanged MbOCA and heat-labile conjugates of MbOCA ('total') in urine has proved to be a reliable and robust method over an 11 year period.

Since the introduction in 1984 of a biological monitoring strategy for MbOCA in the UK, there has been a steady fall in the proportion of workers whose urinary MbOCA levels are above the advisory values. The fall in urinary MbOCA concentrations was associated with improved control measures, better housekeeping and personal hygiene and the use of appropriate personal protective equipment. The introduction of a biological action limit based on the 90th percentile, provided industry with a target to aim for which was reasonably practicable. We have observed that over time the industry in general has responded well to the setting of a biological monitoring value and that this has had the practical effect of helping to reduce MbOCA exposure as measured by urinary MbOCA.

HSC's Advisory Committee on Toxic Substances has recently agreed a framework for the use of biological monitoring and the assignment of biological monitoring guidance values (HSE 1996). A biological monitoring guidance value is set where it is likely to be of practical value and where suitable monitoring methods exist and where there is sufficient information to permit meaningful interpretation of the data. There are two types of guidance value. The health guidance value is set at a level at which there is no indication from the available scientific evidence that the substance is likely to be injurious to health. The second type is the benchmark guidance value. This value is hygiene-based and is set at around the 90th percentile of available validated data, provided by a good cross-sectional study of workplaces with good occupational hygiene practices.

The concept of the 'benchmark' is somewhat different from the approach used in other countries. In the USA the American Conference of Governmental Industrial Hygienists have proposed the use of biological exposure indices which are exposure-based standards (ACGIH 1995) and the Deutsche Forschungsgemeinschaft publish health-based biological tolerance values (DFG 1995). The benchmark is a practical approach which can be used when dose–response data are not available or when, because of the potential toxicity of a substance, it is not possible to conduct laboratory-based volunteer studies to define its disposition or establish a no adverse effect level. HSC's Advisory Committee on Toxic Substances has recommended that the advisory biological monitoring benchmark for MbOCA should be 15 μmol MbOCA per mol creatinine (HSE 1996).

References

ACGIH (1995) *1995–1996 Threshold Limit Values and Biological Exposure Indices* (American Conference of Government Industrial Hygienists, Cincinnati, Ohio).

BAILEY, E., BROOKS, A. G., FARMER, P. B. AND STREET, B. (1993) Monitoring exposure to 4,4′-methylene-bis (2-chloroaniline) through the gas chromatography – mass spectrometry measurement of adducts to haemoglobin. *Environmental Health Perspectives*, 99, 175–177.

COCKER, J. AND WILSON, H. K. (1989) Determination of 4,4′-methylenebis-(2-chloroaniline) in urine. *Clinical Chemistry*, 35, 506.

COCKER, J., BOOBIS, A. R. AND DAVIES, D. S. (1988) Determination of the N-acetyl metabolites of 4,4′ methylenedianiline and 4,4′ methylenebis-(-2 chloroaniline) in urine. *Biomedical and Environmental Mass-Spectrometry*, 17, 161–167.

COCKER, J., BOOBIS, A. R., WILSON, H. K. AND GOMPERTZ, D. (1990) Evidence that a β-N-glucuronide of 4,4′-methylenebis-(2-chloroaniline) (MbOCA) is a major urinary metabolite in man: implications for biological monitoring. *British Journal of Industrial Medicine*, 47, 154–161.

COCKER, J., NUTLEY, B. P. AND WILSON, H. K. (1994) A biological monitoring assessment of exposure to methylene dianiline in manufacturers and users. *Occupational and Environmental Medicine*, 51, 519–522.

DFG (1995) *MAK-und BAT- Werte-Liste 1995*. Mitteilung 31, Deutsche Forschungsgemeinschaft (VCH, Weinheim).

DUCOS, P., MAIRE, C. AND GAUDIN, R. (1985) Assessment of occupational exposure to 4,4′-methylenebis-(2-chloroaniline) (MOCA) by a new sensitive method for biological monitoring. *International Archives of Occupational and Environmental Health*, 55, 159–167.

GRISTWOOD, W., ROBERTSON, S. M. AND WILSON, H. K. (1984) Determination of 4,4′-methylenebis-(2-chloroaniline) in urine by electron-capture gas chromatography. *Journal of Analytical Toxicology*, 8, 101–105.

HOTCHKISS, S. A. M., HEWITT, P. AND CALDWELL, J. (1993) Percutaneous absorption

of 4,4'-methylene-bis-(2-chloroaniline) and 4,4'-methylenedianiline through rat and human skin in-vitro. Toxicology in-vitro, **7**, 141–148.

HSC (1987) Health and Safety Commission News Release 10 June 1987, following review by the HSC's Advisory Committee on Toxic Substances.

HSE (1996) EH40/96 Occupational Exposure Limits (Health and Safety Executive).

JAFFE, M. (1886) Measurement of creatinine using picric acid. Zeitschrift für Physiologische Chemie, **10**, 391–400.

JEDRZEJCZAK, K. AND GAIND, V. S. (1992) Determination of 4,4'-methylenebis-(2-chloroaniline) in urine using capillary gas chromatography and negative ion chemical ionisation mass spectrometry. Analyst-London, **117**, 1417-1420.

KADERLIK, K. R., TALASKA, G., DE BORO, G., OSORIA, A. M. AND KADLUBAR, F. F. (1993) 4,4'-Methylene-bis-(2-chloroaniline)-DNA adduct analysis in human exfoliated cells by ^{32}P-postlabelling. Cancer Epidemiology, Biomarkers and Prevention, **2**, 63–69.

LINCH, A. L O'CONNOR, G. B., BARNES, J. R., KILLIAN, A. G. AND NEELD, W. E. (1971) Methylene-bis-ortho-chloroaniline (MOCA): Evaluation of hazards and exposure control. American Industrial Hygiene Association Journal, **32**, 802–819.

LOWRY, L. K. AND CLAPP, D. E. (1992) Urinary 4,4'-methylenebis-(2-chloroaniline) (MbOCA); a case for biological monitoring. Applied Occupational & Environmental Hygiene, **7**, 593–598.

McKERRELL, P. J., SAUNDERS, G. A. AND GEYER, R. (1987) Determination of 4,4'-methylenebis-(2-chloroaniline) in urine by high-performance liquid chromatography. Journal of Chromatography, **408**, 399–401.

McQUEEN, C. A. AND WILLIAMS, G. M. (1990) Review of the genotoxicity and carcinogenicity of 4,4'-methylenedianiline and 4,4'-methylenebis-(2-chloroaniline). Mutation Research, **239**, 133–142.

OSORIO, A. M., CLAPP, D., WARD, E., WILSON, H. K. AND COCKER, J. (1990) Biological monitoring of a worker acutely exposed to MbOCA. American Journal of Industrial Medicine, **18**, 557–589.

SMITH, A. M. AND WOODWARD, K. (1983) 4,4'-methylenebis-(2-chloroaniline) (MbOCA) Toxicity Review (HMSO, London).

THOMAS, J. D. AND WILSON, H. K. (1984) Biological monitoring of workers exposed to 4,4'-methylenebis-(2-chloroaniline) (MbOCA). British Journal of Industrial Medicine, **41**, 547–551.

WARD, E., SMITH, A. B. AND HALPERIN, W. (1987) 4,4'-Methylenebis-(2-chloroaniline): an unregulated carcinogen. American Journal of Industrial Medicine, **12**, 537–549.

WILL, W., GOSSLER, K. AND RAITHEL, H. J. (1981) Quantitative determination of 4,4'-methylenebis-(2-chloroaniline) (MOCA) in urine by high-pressure liquid chromatography. Arbeitsmedizin Sozialmedizin Preventivmedizin, **16**, 201–203.