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Monitoring for occupational exposure to 4,4′-methylenebis(2-chloroaniline) by gas chromatographic-mass spectrometric analysis of haemoglobin adducts, blood, plasma and urine

G.T. Vaughan[a,*], R.S. Kenyon[b]

[a]*Centre for Advanced Analytical Chemistry, CSIRO Division of Coal and Energy Technology, Private Mail Bag 7, Bangor, NSW 2234, Australia*

[b]*Workcover Authority of NSW, Bangor, NSW 2234, Australia*

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Abstract

The feasibility of using plasma, blood and haemoglobin adducts for monitoring occupational exposure to the suspected human carcinogen 4,4′-methylenebis(2-chloroaniline) (MOCA) was investigated. A method utilising capillary gas chromatography–negative-ion chemical-ionisation mass spectrometry (GC–MS) for the determination of pentafluoropropionyl (PFP) derivatives of MOCA, released by alkaline hydrolysis from protein adducts and conjugates, was both sensitive and selective. When selected ion monitoring was used, sub-femtomole amounts of PFP-MOCA could be measured. The detection limit for haemoglobin adducts of MOCA was below 10 fmol/g Hb, well below the levels found for occupationally exposed individuals. Capillary GC with electron-capture detection also had the required sensitivity for the determination of MOCA in blood samples, however, the presence of interfering compounds in some samples limited its use. The levels of MOCA in the blood and urine of five individuals who were exposed to MOCA during the manufacture of polyurethane elastomers were determined by the GC–MS method. The MOCA concentrations for the various blood fractions and urine were within the following ranges: haemoglobin adducts, 0.73–43.3 pmol MOCA/g Hb; plasma alkaline hydrolysate, 0.05–22.0 nmol/l; whole blood, 0.13–17.4 nmol/l; urine, 4.5–2390 nmol/l. Because the products of MOCA in the blood reflect metabolic activation of MOCA and integrate exposure over a period of weeks, the use of blood samples for monitoring exposure to MOCA offers advantages over the currently used urinary MOCA measurements.

Keywords: 4,4′-Methylenebis(2-chloroaniline)

1. Introduction

4,4′-Methylenebis(2-chloroaniline) (MOCA) (Fig. 1) is a suspected human carcinogen that is widely used as a cross-linking agent in the manufacture of

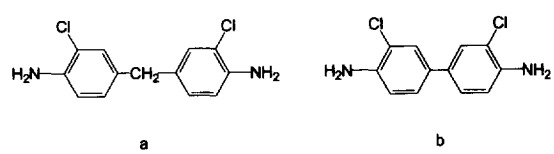

Fig. 1. Chemical structures of (a) 4,4′-methylenebis(2-chloroaniline) and (b) 3,3′-dichlorobenzidine (internal standard).

*Corresponding author.

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polyurethane elastomers. It is an aromatic amine, structurally related to benzidine, a human bladder carcinogen. MOCA is mutagenic [1,2], and carcinogenic to rats, mice and dogs [3–6].

Because occupational exposure to MOCA occurs mainly through skin absorption [7,8], biological monitoring — in particular the determination of MOCA excreted in the urine — is the main method for monitoring the exposure of workers to MOCA. However, the half-time for the excretion of MOCA in urine was calculated as 23–24 h [9,10] for accidental exposures to high concentrations of MOCA. Measurements of MOCA in urine for exposed workers [8] have shown that large fluctuations can occur throughout the day and from day to day. Therefore, the time at which urine is collected can greatly influence the results obtained. If samples are collected once in several months, as is often the practice, excessive exposures may not be detected.

An alternative to measuring the concentrations of MOCA in urine is to determine the concentrations of haemoglobin adducts of MOCA in the blood. Haemoglobin adducts of aromatic amines are formed by the reaction of hydroxylamine metabolites of amines with a cysteine sulphydryl on haemoglobin [11]. These adducts are stable for the life span of haemoglobin, which in humans is about 120 days. The analysis of haemoglobin adducts has been used for monitoring exposure to a range of aromatic amines, including aniline, *p*-chloroaniline, benzidine, 2-naphthylamine and 4-aminobiphenyl [12–14].

Haemoglobin adducts formed in rats dosed with MOCA have been measured using HPLC with electrochemical detection [15], gas chromatography with electron capture-detection (GC–ECD) [16] and gas chromatography–mass spectrometry (GC–MS) [15,17]. The present work tested the suitability of GC–ECD and GC–MS methods for monitoring workers exposed to MOCA. The concentrations of haemoglobin adducts in these workers were 100 to 1000 times lower than those measured in the animal experiments. This paper reports the optimisation of sample preparation and analysis to measure the haemoglobin adducts of MOCA in workers. Apart from urinary MOCA, very little information is available for the concentrations of MOCA in the body fluids of humans. In this work, the concentrations of MOCA in urine, whole blood, haemoglobin adducts, and plasma of a group of workers exposed to MOCA are reported.

2. Experimental

2.1. Materials

4,4′-Methylenebis(2-chloroaniline) [>98% purity] was obtained from Tokyo Kasei Kogyu (Tokyo, Dapon), 3,3′-dichlorobenzidine from Sigma (St. Louis, MO, USA), 4,4′-methylendianiline from Aldrich (Milwaukee, WI, USA); pentafluoropropionic anhydride from Pierce (Rockford, IL, USA) and acetic anhydride from Fluka (Buchs, Switzerland). Nanograde hexane and ether were supplied by Mallinkrodt. All other chemicals and solvents were of analytical grade.

2.2. Samples

Blood and urine samples were obtained from workers involved in the production of polyurethane elastomers. Blood was obtained by venipuncture and stored in heparinised glass tubes. A 10-ml sample of blood was separated into erythrocytes and plasma by centrifuging at 1000 *g* for 15 min. The erythrocytes were washed three times with isotonic saline and haemolysed by the addition of twice the volume of distilled water and vigorous shaking. After 30 min, cellular debris was removed by centrifuging at 10 000 *g* for 30 min. The haemolysate was transferred to Spectra/Por 4 dialysis tubing (25 mm flat width, 12–14 000 M_r cut-off; Spectrum, Houston, TX, USA) and dialysed against distilled water for 48 h with changes of water at 4 and 24 h.

2.3. Determination of haemoglobin adducts

The haemoglobin solution from a 10-ml sample of blood was transferred from the dialysis bag into a weighed 50-ml screw-capped culture tube with a Teflon-lined lid and then reweighed to determine the approximate volume. A sub-sample (300 μl) was taken for the determination of haemoglobin by the Drabkin method (Sigma Kit 525A). To the remainder

of the dialysed haemoglobin solution, an internal standard (10 ng of 3,3′-dichlorobenzidine–50 μl 200 ng DCB/ml methanol) and 5 *M* NaOH to give a final concentration of 0.1 *M* were added. The tubes were gently mixed at 20°C for 3 h. The parent amines released from their haemoglobin adducts were extracted twice with 15 ml of hexane using gentle agitation to mix the phases (vigorous mixing leads to the formation of difficult emulsions). Any emulsions formed were broken by freezing and thawing. The extracts were combined, hexane was evaporated under nitrogen and the residue stored at −20°C until analysis.

2.4. MOCA in whole blood and plasma

Samples of blood and plasma (5 ml), with an internal standard (DCB) added, were hydrolysed by the addition of an equal volume of 1 *M* NaOH and reaction at 95°C for 2 h. The procedure used for extraction and analysis of MOCA was the same as for the analysis of MOCA released from haemoglobin adducts. Ultrafiltration of a 2 ml sample of plasma (22 nmol MOCA/L) was carried out in a Centricon-10 (10000 M_r cut-off filter) microconcentration tube (Amicon, Danvers, MA) centrifuged at 2000 *g* for 30 min. The retentate was washed from the filter with Milli-Q water. MOCA in the ultrafiltrate and the retentate was determined by the same procedure used for the determination of MOCA in plasma.

2.5. Derivatisation of aromatic amines

Aromatic amine standards and dried extracts were derivatised with 1% pentafluoropropionic anhydride in dry hexane (200 μl) at 50°C for 15 min. Hexane and excess derivatising agent were removed by evaporation under nitrogen. Samples were dissolved in 100 μl dry ethyl acetate before analysis by GC–MS or GC–ECD.

2.6. Determination of MOCA in urine

Urine samples from workers were collected in glass urine jars and stored at −20°C until analysis. An internal standard (500 ng of 3,3′-dichlorobenzidine or 4,4′-methylenedianiline) and 2 ml of 2 *M* NaOH were added to a 5 ml sample of urine. The sample was mixed by vortexing and heated in a dry-block at 95°C for 1 h. The alkaline hydrolysate was cooled to room temperature and extracted twice with 2 ml of hexane. The hexane phase was evaporated under nitrogen, reacted with pentafluoropropionic anhydride and analysed by GC–ECD or GC–MS. Creatinine concentrations in the urine samples were determined by a colorimetric procedure based on the Jaffé reaction (Sigma Kit No. 555-A). The results for MOCA in urine samples were normalised for creatinine concentration.

2.7. Gas chromatography

GC was carried out on a Varian 3400 gas chromatograph fitted with a temperature-programmable on-column injector, and an electron-capture (^{63}Ni) detector. The electron-capture detector was interfaced with a Waters 840 Data Station for the storing and integrating of the chromatograms. The carrier gas was hydrogen at 2 ml/min. The electron-capture detector was maintained at 350°C and the flow-rate of the nitrogen make-up gas was 38 ml/min.

For the separation of aromatic amines, a 30 m × 0.25 mm I.D. SE-30 column (Alltech) with a phase thickness of 0.25 μm. A retention gap (2 m × 0.25 mm I.D. deactivated fused-silica column) was connected to the column with a direct connect (Alltech) capillary connector. The retention gap was replaced when peak shape deteriorated. The samples were injected at 50°C into the on-column injector which was programmed to rise to 230°C at 100°C/min. The column was held at 50°C for 1 min, raised to 200°C at 50°C/min, held at that temperature for 10 min and then raised to 230°C at 10°C/min. Retention times: N,N′-dipentafluoropropionyl-MOCA, 15.6 min; N,N′-dipentafluoropropionyl-3,3′-dichlorobenzidine, 14.0 min; N,N′-dipentafluoropropionyl-4,4′-methylenenedianiline, 17.29 min.

2.8. Gas chromatography–mass spectrometry

GC–MS was performed on a VG Trio-1 S mass spectrometer (VG Instruments, Manchester) inter-

faced to a Hewlett Packard HP5890A Series II gas chromatograph fitted with a split/splitless injector operating in the splitless mode. The inlet purge off time was set at 30 s. Electron energy was 70 eV, emission current 400–450 μA, electron multiplier voltage 800 mV. Commercially pure methane was used as the chemical ionization (CI) energy moderator and collisional stabilisation gas (ion source pressure 107 Pa). Samples analysed by GC–MS were injected onto a SE-54 (Alltech) capillary column (30 m × 0.25 mm I.D., 0.25 μm phase thickness) with a 2 m × 0.25 mm I.D. deactivated fused-silica retention gap, connected to the column with a Direct Connect (Alltech) capillary connector. Helium was the carrier gas at 0.9 ml/min. The initial column temperature of 60°C was held for 1 min and programmed to rise to 320°C at 50°C/min. For the analysis of trace amounts of aromatic amines, selected-ion monitoring was used and the following ions were monitored: *m/z* 524 (N,N′-dipentafluoropropionyl-3,3′-dichlorobenzidine t_R =8.04 min); *m/z* 538 (N,N′-dipentafluoropropionyl-4,4′-methylenebis- (2-chloroaniline), t_R =8.19 min); *m/z* 434 (N-pentafluoropropionyl-N-acetyl-4,4′-methylenebis-(2-chloroaniline), t_R =9.8 min).

2.9. Negative-ion spectra of pentafluoropropionyl (PFP) derivatives of aromatic amines

4,4′-Methylenebis(2-chloroaniline)–(PFP)$_2$ CIMS (methane GC)

m/z (relative intensity): 540 (65), 538 (100) $[M-HF]^-$, 524 (10), 522 (24) $[M-HCl]^-$, 504 (11), 502 (25), 466 (4), 219 (3).

3,3′-Dichlorobenzidine–(PFP)$_2$: CIMS (methane GC)

m/z (relative intensity): 526 (68), 524 (100) $[M-HF]^-$, 508 (19) $[M-HCl]^-$, 490 (17), 488 (42), 472 (15), 219 (6).

3. Results and discussion

The concentration of MOCA in body fluids is usually determined by measuring the amounts of both free MOCA and MOCA released from metabolic products after hydrolysis with acid or base. In urine, the major product of MOCA is the β-N-glucuronide [18]. This conjugate can be hydrolysed by strong base to release MOCA. In the blood, aromatic amines form adducts with haemoglobin by the formation of sulphinic acid amides with cysteine residues of haemoglobin [11]. These adducts are susceptible to hydrolysis by weak bases, releasing the parent aromatic amine.

Several analytical techniques have been used to measure MOCA in the urine of exposed workers at concentrations measured in nmol/l [19]. However, many of these techniques are not sensitive enough for the determination in blood of MOCA which is present at concentrations two orders of magnitude lower. Two techniques, GC–ECD and GC–MS with negative ion detection, were found to be sensitive enough to measure the concentrations of MOCA in blood.

3.1. Gas chromatography with electron-capture detection

The chromatographic properties of aromatic amines and their response to ECD can be improved by the formation of perfluorinated derivatives. In particular, pentafluoropropionyl (PFP) derivatives of aromatic amines had improved peak shapes and enhanced detection by ECD and negative-ion MS. However, at low concentrations, the PFP derivatives of some aromatic amines were found to degrade at different rates over several hours. Therefore, derivatives were prepared immediately before analysis in order to avoid differential recoveries of the aromatic amines present in the samples. Improved chromatography was achieved by evaporating the sample under nitrogen to remove excess derivatising agent. If this was not carried out, a large tailing peak at the beginning of the chromatogram caused subsequent peaks to broaden.

Both SE-30 and SE-54 capillary columns gave good resolution of the aromatic amines and were suitable for the analysis of MOCA in urine and blood. The use of cold on-column injection and the addition of a 2-m retention gap resulted in better peak shape than was achieved using a flash evaporation injector. When helium carrier gas was replaced with hydrogen there was also an improvement in the resolution of peaks.

Less than 100 fg of diPFP-MOCA per 1 μl injection was detectable by capillary-column GC with ECD, which was sensitive enough for the measurement of MOCA in the urine and haemoglobin adducts of workers with low levels of exposure. Chromatograms for the determination of MOCA in urine (Fig. 2) showed little interference from other compounds in urine. However, when GC–ECD was used for the determination of MOCA released from haemoglobin adducts (Fig. 3), interferences from other compounds present in the blood extracts were a problem. In the absence of interfering peaks, good correlations between GC–ECD and MS methods were found. However, the chance of false positives limits the utility of this technique for determination of haemoglobin adducts. It may be possible to remove interfering compounds by introducing further clean-up procedures. The need for lengthy sample preparation can be overcome by taking advantage of the increased specificity of mass spectrometric detection.

3.2. Gas chromatography–mass spectrometry

Initially, positive-ion electron-impact mass spectrometry was used because of its wider availability and more common use. The positive-ion electron-impact mass spectra of N,N′-dipentafluoropropionyl

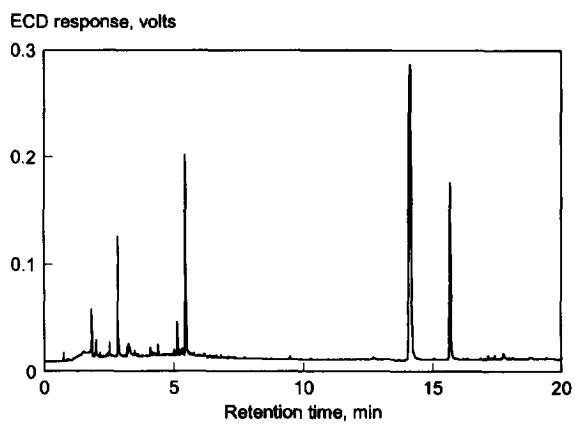

Fig. 2. Determination of MOCA in urine by GC. Column: 30 m × 0.25 mm SE-30 capillary (phase thickness 0.25 μm), 2-m retention gap. Temperature program: 50°C (1.5 min) to 200°C at 50°C/min, held at 200°C for 10 min, then raised to 230°C at 10°C/min. Detector: electron-capture detector. Injector: on-column. Retention times: diPFP-MOCA, 15.6 min; diPFP-DCB, 14.0 min (0.9 ng injected).

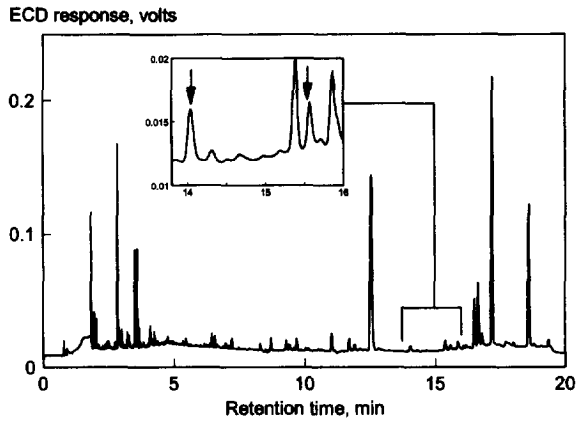

Fig. 3. Determination of haemoglobin adducts of MOCA by GC–ECD. For conditions see Fig. 2. The inset shows the area of the chromatogram where diPFP-DCB (t_R = 14.0 min) and diPFP-MOCA (t_R = 15.6 min) occur.

MOCA (diPFP-MOCA) had a molecular ion at m/z 558, a fragment ion at m/z 523 $[M-Cl]^+$ and a base peak at m/z 250. By using selected-ion monitoring (SIM) for the fragment ion at m/z 523, 10 pg of diPFP-MOCA was detectable. This is sensitive enough for the determination of MOCA in the urine but not for the determination of haemoglobin adducts in workers with moderate exposure to MOCA. Bailey et al. [17] reported the use of positive-ion electron-impact mass spectrometry for the determination of haemoglobin adducts from rats exposed to MOCA by intraperitoneal injection. They reported a detection limit of 10 pmol/g Hb, based on the use of a 50-mg sample of haemoglobin. A similar detection limit was reported for the determination of haemoglobin adducts of 4,4′-methylenedianiline using similar methods [20]. Most of the workers in the present study had levels of haemoglobin adducts that were below this detection limit.

Higher sensitivity for the mass spectrometric measurement of diPFP-MOCA was obtained by using chemical ionisation and negative-ion detection. The mass spectra for aromatic amines, with methane used as the reagent gas, had fewer fragment ions than the corresponding positive-ion electron-impact spectra. The most abundant ions in these spectra (m/z 538 for diPFP-MOCA and m/z 524 for diPFP-DCB) are formed by the loss of HF from the molecular ion. With negative-ion chemical ionisation

mass spectrometry the detection limit for diPFP-MOCA was 50 fg with a signal-to-noise ratio of 5 to 1.

Because of the high specificity of this type of detection, the chromatographic conditions used for separation of the analytes do not need to be as rigorous as for ECD. Therefore, the time required for analysis (9 min) was about half that required for GC–ECD analysis. A typical analysis for MOCA released from haemoglobin adducts and with DCB as an internal standard is shown in Fig. 4. Under the chromatographic conditions used, the peaks are still well resolved, even though the derivatives of MOCA and DCB are separated by only 9 s. In the extracts from workers' blood, MOCA was typically the only peak in the mass chromatogram at *m/z* 538. At *m/z* 524, there were two peaks: one corresponding with DCB; the other with the same retention time as MOCA. The latter peak, which is due to a minor fragment ion of MOCA $[M(540)-HCl]^-$ aids in the identification of the MOCA peak. The internal standard, 3,3′-dichlorobenzidine, used for this work enabled a direct comparison to be made between GC–MS and GC–ECD measurements. A better internal standard for GC–MS is $[^2H_6]$MOCA which has been synthesised by the MRC Toxicology Unit [17].

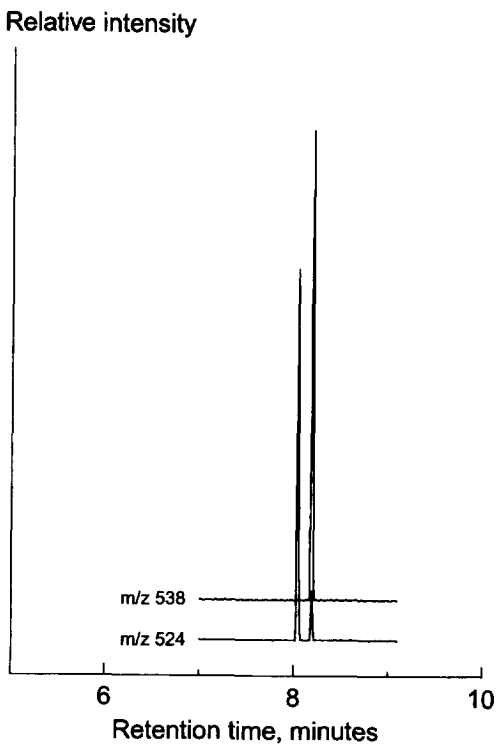

Fig. 4. Determination of haemoglobin adducts of MOCA by GC–MS. A sample of blood from a worker exposed to MOCA was analysed for haemoglobin adducts of MOCA, with DCB added as an internal standard. Chromatographic conditions: 30 m × 0.25 mm SE-30 capillary column (phase thickness, 0.25 μm), 2-m retention gap. Temperature program: 60°C for 1 min, then 50°C/min to 320°C. Retention times: diPFP-DCB (*m/z* 524), 8.04 min (60 pg injected), diPFP-MOCA (*m/z* 538), 8.19 min.

3.3. Determination of haemoglobin adducts of MOCA

The method used to isolate and analyse haemoglobin adducts of aromatic amines involved the haemolysis of erythrocytes, dialysis of the released haemoglobin, the addition of an internal standard (DCB), hydrolysis of haemoglobin adducts with 0.1 *M* NaOH, and determination of the released MOCA by GC–MS. The precision of this assay was determined by the analysis of six replicates of haemoglobin adduct measurements from a single blood sample. The relative standard deviation of these measurements was 8.7%. The limit of detection of this procedure is below 10 femtomole adduct/g Hb for a 10-ml blood sample, which is more than ten times lower than the lowest concentration of haemoglobin adducts that was measured in exposed workers.

The concentrations of haemoglobin adducts in the blood of five workers involved in the production of polyurethane elastomers are shown in Table 1. The haemoglobin adducts ranged from 0.73 pmol/g for a worker carrying out general duties in a factory to 43.3 pmol/g for a worker involved in mixing MOCA with a prepolymer. The lower values are of the same order as haemoglobin adducts of 4-aminobiphenyl that were found for non-smokers (0.19 ± 0.08 pmol/g Hb) and smokers (0.91 ± 0.28 pmol/g Hb) [11]. The upper values for MOCA adducts are comparable to the concentrations of haemoglobin adducts of 4,4′-methylenedianiline (MDA) that were reported for a worker by Bailey et al. [20]. They found a

Table 1
MOCA in blood fractions and urine of exposed workers

Worker	Whole blood (nmol/l)	Plasma (nmol/l)	Urine (nmol/l)	Haemoglobin adducts (pmol/g Hb)	
				Dialysed haemoglobin	Undialysed haemoglobin
A	0.13	0.05	4.5	0.73	0.78
B	1.07	0.39	62.1	5.81	5.17
C	1.61	0.56	65.5	7.54	6.72
D	3.56	2.96	132.7	14.34	11.71
E	17.37	21.95	2390	43.30	43.25

concentration of 10.88 pmol MDA/g Hb but the concentration of adducts of N-acetyl MDA was higher (25.33 pmol/g Hb).

One of the in vivo reactions of aromatic amines is N-acetylation, catalysed by the enzyme N-acetyltransferase. The acetylation reaction deactivates many xenobiotics, but for aromatic amines such as benzidine, acetylation was found to enhance binding to DNA and to haemoglobin [13,21]. When rats were exposed to benzidine, the ratio of N-acetyl benzidine to benzidine bound to the haemoglobin was 10:1. By contrast, haemoglobin adducts of N-acetyl MOCA measured in the blood of the workers described in the present work were lower in concentration than those of the diamine with the ratio of N-acetyl MOCA to MOCA between 0.016 to 0.05. The concentrations of N-acetyl metabolites in urine of workers follow the same trend as the haemoglobin adducts; the ratio of N-acetyl MDA to MDA was 1-24, and the ratio of N-acetyl MOCA to MOCA in the urine was 0.091 to below 0.005 [22]. Therefore, it appears that N-acetylation of MOCA is not an important reaction for the detoxificaton of MOCA or for its activation into a species likely to bind to DNA.

Green et al. [12] noted that dialysis of haemoglobin solutions before the determination of haemoglobin adducts of 4-ABP was required to remove unbound 4-ABP. However, the amounts of MOCA released from dialysed haemoglobin solutions for most measurements were only slightly higher than those released from undialysed solutions (Table 1). The differences between the two measurements were of the order of those found for replicate analyses of a single sample. Therefore, if haemoglobin adducts of MOCA are the only adducts of interest, then it would not be necessary to carry out time-consuming dialysis of the samples.

3.4. Partitioning of MOCA and its products in blood

Blood samples from five workers were investigated to determine the amounts of MOCA and its hydrolysable products in various blood fractions (Table 1). These measurements were made after alkaline hydrolysis (0.5 *M* NaOH, 95°C, 2 h) to release MOCA from adducts and conjugates. The concentrations of MOCA in whole blood, measurements which include haemoglobin adducts, were between 0.127 and 17.37 nmol/l and were 37 to 196 times lower than the concentrations in urine samples collected at the same time as the blood samples. Of the total amount of MOCA measured in blood samples, haemoglobin adducts represented 34.8 to 82.6%.

The concentrations of MOCA in plasma samples ranged from 0.45 to 21.95 nmol/l, however, the exact form in which this MOCA occurs is uncertain. Other mutagenic compounds, including 4-aminobiphenyl and aflatoxin B_1, form adducts with plasma proteins such as serum albumin [11]. Methods for the determination of methylenedianiline in plasma have recently been published [23]. Highest recoveries of methylenedianiline were obtained after alkaline hydrolysis, presumably to release methylenedianiline from protein adducts or conjugates. An indication of the nature of MOCA in plasma was obtained by ultrafiltration of the most concentrated sample (21.95 nmol MOCA/l) with a 10 000 M_r

cut-off filter. Only 15% of the MOCA was located in the ultrafiltrate, the remainder being associated with the high molecular weight fraction, possibly as hydrolysable adducts to plasma proteins.

4. Conclusions

The most widespread method for monitoring the exposure of workers to MOCA is to measure the concentrations of MOCA excreted in the urine. The half-life for MOCA in urine is approximately one day and urinary monitoring gives an indication of the exposure of workers to MOCA over the few days before a sample is collected. By contrast, information on exposure to MOCA over the preceding 120 days can be gained from the analysis of haemoglobin adducts. If workers are only tested infrequently, the measurement of haemoglobin adduct concentrations will give a more useful estimate of worker exposure over the period between tests than will urinary monitoring. In addition, haemoglobin adduct measurements are more relevant to the adverse health effects of exposure to MOCA because they reflect the metabolic activation of MOCA into species that have the potential to react with DNA. In addition to haemoglobin adduct and urinary MOCA measurements, the determination of MOCA in plasma may be of use for future monitoring studies as it may have a half-life between that of haemoglobin adducts and urinary MOCA. When all three measurements are used together, it may be possible to gain information on the time that has elapsed since a high level of exposure has occurred.

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