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# A Method for Routine Analysis of Urinary 4,4'-methylenebis (2-chloroaniline) by Gas Chromatography-Electron Capture Detection

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**Abstract: A Method for Routine Analysis of Urinary 4,4'-methylenebis (2-chloroaniline) by Gas Chromatography-Electron Capture Detection: Akito Takeuchi, *et al*. Osaka Occupational Health Service Center, Japan Industrial Safety and Health Association—Objectives:** The purpose of this study was to develop a simple and cost-effective method for the determination of urinary 4,4'-methylenebis (2-chloroaniline) (MBOCA) by gas chromatography-electron capture detection (GC-ECD) for biological monitoring of occupational exposure to MBOCA. **Methods:** MBOCA was prepared by liquid-liquid extraction after alkaline hydrolysis, derivatized with *N*-methyl-bis (trifluoroacetamide) and then analyzed using GC-ECD. The proposed method was validated in accordance with the US Food and Drug Administration guidance. **Results:** The calibration curve showed linearity in the range 1–100 $\mu$g/*l*, with a correlation coefficient of >0.999. The limits of detection and quantification were 0.3 $\mu$g/*l* and 1 $\mu$g/*l*, respectively. The recovery was 94−99%. Intraday accuracy, expressed as the deviation from the nominal value, was 90.5−100.3%, and intraday precision, expressed as the relative standard deviation, was 0.3−2.4%. Interday accuracy and precision were 87.8−100.2% and 0.3−4.1%, respectively. **Conclusions:** The proposed method is a simple and cost-effective method suitable for routine analyses and could be useful for biological monitoring of occupational exposure to MBOCA.
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**Key words:** 4,4'-Methylenebis (2-chloroaniline), Biological monitoring, Gas chromatography-electron capture detection, Urine

The 4,4'-methylenebis (2-chloroaniline) (MBOCA) is widely used as a curing agent for urethane elastomers and as epoxy resin and epoxy urethane hardeners[1]. MBOCA has been classified as a Group 2A (probably carcinogenic to humans) compound by the Japan Society for Occupational Health (JOH)[2]. Moreover, the JOH adopted an occupational exposure limit based on biological monitoring of urinary MBOCA (50 $\mu$g/g creatinine) after hydrolysis (total MBOCA)[1, 2].

In 2008, an expert committee of the Ministry of Health, Labour and Welfare of Japan reported that urinary total MBOCA should be measured in the periodic health checkups of workers exposed to MBOCA[3]. Therefore, development of a simple method suitable for routine analyses is of great interest.

Several methods have been reported for the determination of MBOCA in urine[4–8]. However, each of these methods has disadvantages, such as the requirement of complex, costly and time-consuming sample preparation procedures including derivatization or solid-phase extraction (SPE). The aim of the present study was to develop and validate a simple and cost-effective method for the determination of total MBOCA in urine for routine analysis. To achieve this aim, we used liquid-liquid extraction (LLE) to reduce the cost and time and derivatization with *N*-methyl-bis (trifluoroacetamide) (MBTFA) and gas chromatography-electron capture detection (GC-ECD) commonly used in many clinical laboratories related to occupational health to obtain the sensitivity required for biological monitoring.

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## Materials and Methods

*Materials*

MBOCA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan); 3,3'-dichlorobenzidine (DCB, 5,000 μg/ml in methanol), used as an internal standard (IS), was purchased from Supelco (Bellefonte, PA, USA); and MBTFA (98–100%) was purchased from Thermo Fisher Scientific (Bellefonte, PA, USA). All other reagents were of analytical grade. A standard stock solution of MBOCA was prepared in acetonitrile (1 g/l) and stored at 4°C. MBTFA was used without any dilution. Urine samples were collected from five healthy adult volunteers. These volunteers were not occupationally exposed to MBOCA. Informed consent was obtained from the volunteers before collection of urine. This study was approved by the Ethics Committee of the Japan Industrial Safety and Health Association (approval number 1).

*Instruments*

The GC-ECD system used was a 7890A gas chromatograph equipped with a micro-electron capture detector (Agilent Technologies, Palo Alto, CA, USA). The column was a 30 m × 0.25 mm ID Equity-5 capillary column with a 0.25-μm film thickness (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The temperature of the injection port was set at 250°C. The detector was maintained at 300°C with a makeup gas ($N_2$) flowing at 30 ml/min. The oven temperature was set at 100°C for 1 minute and then increased to 300°C at a rate of 10°C/min. Samples (1 μl) were injected in pulsed splitless mode (pulse pressure, 25 psi; pulse time, 1 minute; purge activation time, 0.9 minutes).

*Sample preparation*

Urine (5 ml) was placed in a glass test tube. An IS (DCB, 5 mg/l, 50 μl) and 2 M NaOH (2 ml) were added to the tube, which was vortexed (10 seconds) and then heated in a boiling water bath for 1 hour. The alkaline hydrolysate was cooled to room temperature and then extracted with toluene (1 ml) by shaking for 2 minutes. The sample was centrifuged at 3,000 rpm for 10 minutes, after which a 0.5-ml aliquot of the toluene layer was transferred to an auto-sampler vial for GC analysis. MBTFA (10 μl) was added to the vial, which was closely capped and then vortexed (10 seconds) and allowed to stand for 30 minutes at room temperature (25°C). A 1-μl aliquot of the sample was injected into the GC-ECD system.

*Method validation*

The proposed method was validated in accordance with the US Food and Drug Administration (FDA) guidance[9]. To plot the calibration curve, urine samples spiked with MBOCA at seven concentrations (matrix-matched standard) ranging from 1 to 100 μg/l were prepared in triplicate, and the prepared samples were analyzed using the procedure employed for sample preparation. The calibration curve was obtained by plotting the peak area ratio of the trifluoroacetyl derivative of MBOCA (MBOCA-TFA) to IS (DCB-TFA) against their respective concentrations. The reproducibility, which was defined as the precision, of the developed method was evaluated by analyzing urine samples containing three different concentrations (1, 25 and 100 μg/l) of MBOCA on the same day (five replicates; intraday reproducibility) and over three consecutive days (five replicates; interday reproducibility). Recovery was determined by comparing the MBOCA-TFA/DCB-TFA peak area ratio in spiked urine samples with those of standard solutions. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the amounts of MBOCA in urine that corresponded to 3 and 10 times the baseline noise, respectively.

## Results and Discussion

*Sample preparation and GC-ECD analysis*

The human urinary metabolites of MBOCA are MBOCA itself and the *N*-acetyl, *N,N'*-diacetyl and *N*-glucuronide conjugates. Total MBOCA is the sum of free MBOCA and MBOCA released from the metabolites by hydrolysis[1]. Therefore, we carried out hydrolysis according to the procedure employed in previous studies[5].

Several methods for determination of MBOCA in urine have been reported, including gas chromatography with mass spectrometry (GC-MS)[4, 5], high-performance liquid chromatography (HPLC) with ultraviolet detection (UV)[6], electrochemical detection (ECD)[7], and tandem mass spectrometry (MS/MS)[8]. The GC methods required a derivatization procedure with pentafluoropropionic anhydride. These derivatization procedures are complex and time-consuming because excess derivatization reagent and its acidic by-product need to be removed before analysis in order to prevent damage to the GC column. Although sample preparation for the HPLC method is easier than that for the GC method, it includes an SPE procedure. The SPE procedure is costly because SPE cartridges should be disposed after usage.

To overcome these disadvantages, we adopted the LLE procedure because it could reduce the cost and time compared with the SPE procedure. Moreover, derivatization with MBTFA and ECD detection were used to obtain the sensitivity required for biological monitoring. The reaction scheme for MBOCA and DCB with MBTFA is presented in Fig. 1. In

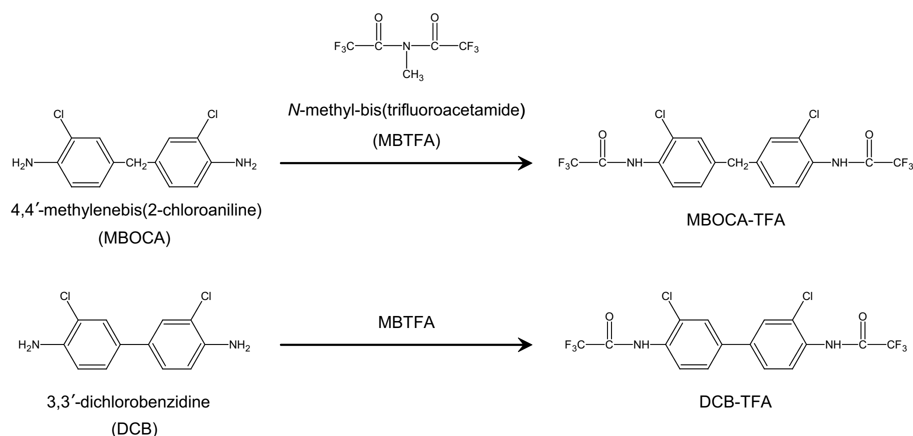


**Fig. 1.** Reaction scheme for 4,4'-methylenebis (2-chloroaniline) and 3,3'-dichlorobenzidine with *N*-methyl-bis (trifluoroacetamide).

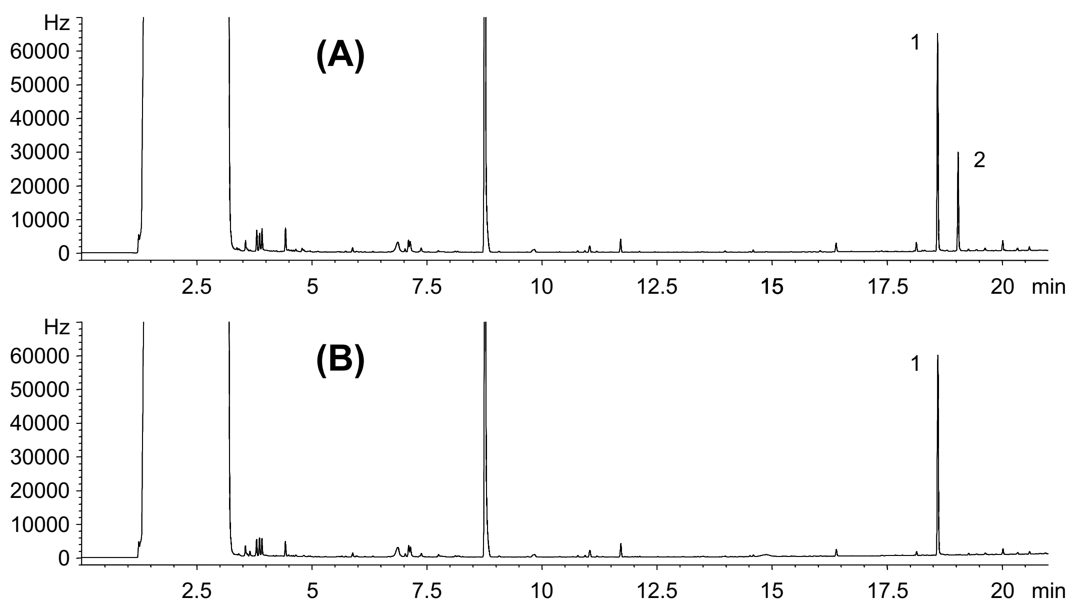


**Fig. 2.** Chromatograms of (A) standard spiked urine (25 $\mu$g/*l*) and (B) blank urine. The peaks of trifluoroacetyl derivative of (1) 3,3'-dichlorobenzidine (DCB-TFA) and (2) 4,4'-methylenebis (2-chloroaniline) (MBOCA-TFA) are indicated.

the derivatization reaction with MBTFA, water in the reaction mixture often interferes with the reaction. Therefore, toluene was chosen as the extraction solvent because it is a hydrophobic solvent with a higher recovery rate. The reaction mixture was injected directly onto a GC column, because the MBTFA itself and the principal by-product of the derivatization reaction were stable and volatile and did not interfere with chromatography. The derivatization conditions, such as the reaction temperature and reaction time, were optimized. The derivatization reaction with MBTFA was completed within 30 minutes at room temperature (25°C). Typical chromatograms of standard spiked urine and blank urine are shown in Fig. 2. The chromatographic peaks corresponding to MBOCA-TFA and DCB-TFA were identified by using a mass spectrometer.

*Validation*

The calibration curve showed linearity in the range 1–100 $\mu$g/*l*, with a correlation coefficient of >0.999. LOD and LOQ were 0.3 $\mu$g/*l* and 1 $\mu$g/*l*, respectively. The recovery was 94–99%. Intraday accuracy, expressed as the deviation from the nominal value, was 90.5–100.3%, and intraday precision, expressed as the relative standard deviation (RSD), was 0.3–2.4%.

**Table 1.** Intra- and interday coefficients of variation of the proposed method

| Spiked urine concentration (μg/l) | Recovery (n=5) | | Intraday (n=5)[a] | | | Interday (n=15)[b] | | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± SD (%) | RSD (%) | Mean ± SD (μg/l) | RSD (%) | Accuracy (%) | Mean ± SD (μg/l) | RSD (%) | Accuracy (%) |
| 1 | 99 ± 2.4 | 2.4 | 0.90 ± 0.02 | 2.4 | 90.5 | 0.88 ± 0.04 | 4.1 | 87.8 |
| 25 | 95 ± 0.5 | 0.5 | 23.52 ± 0.13 | 0.5 | 94.1 | 23.41 ± 0.12 | 0.5 | 93.6 |
| 100 | 94 ± 0.3 | 0.3 | 100.29 ± 0.33 | 0.3 | 100.3 | 100.24 ± 0.30 | 0.3 | 100.2 |

RSD, relative standard deviation. [a] Intraday reproducibility analysis was performed on a single day. [b] Interday reproducibility analysis was performed over three consecutive days in five replicates.

Interday accuracy and precision were 87.8–100.2% and 0.3–4.1%, respectively (Table 1). In comparison with the previously reported methods, the proposed method showed a similar LOD (GC-MS, 1 μg/l; HPLC-UV, 1 μg/l)[4, 6] and LOQ (HPLC-ECD, 6.7 μg/l; HPLC-MS/MS, 1 μg/l)[7, 8].

## Conclusions

We developed and validated a GC-ECD method to determine total MBOCA in urine. The proposed method is a simple and cost-effective method suitable for routine analyses and could be useful for biological monitoring of occupational exposure to MBOCA.

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