or not any functional differences are associated with these differences in NE content remains to be seen. Sulfo-conjugated catecholamines, a major fraction of total catecholamine in the erythrocyte compartment, must also be measured in men and women.

In developing this method, we attempted to use trichloroacetic acid (100 g/L) to burst the cells and remove the erythrocyte proteins from the samples. Although we successfully lysed the cells and removed all erythrocyte proteins, there was a >95% loss of catecholamine, as measured by the recovery of internal standard. Sonication of the erythrocytes to release the free catecholamine also proved unsuccessful, leading to interference in the chromatography.

We recommend this method of extraction and quantification of erythrocyte catecholamines as suitable for routine use in the clinical and research laboratory.

R. J. A. is a Fellow, Stanley J. Sarnoff Endowment for Cardiovascular Science.

References

Determination of 4,4'-Methylenebis(2-chloroaniline) in Urine by Liquid Chromatography with Ion-Paired Solid-Phase Extraction and Electrochemical Detection

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This highly specific and sensitive method for measuring urinary 4,4'-methylenebis(2-chloroaniline) (MBOCA) involves liquid chromatography with electrochemical detection. Before chromatography, urine samples are prepared by ion-paired solid-phase extraction on a disposable octadecylsilica column with acidic methanol solution containing 1-heptanesulfonic acid. This enhances the specificity of the method. Mean overall recovery ranged from 97.1% to 99.5% at added MBOCA concentrations of 20 and 100 μg/L in urine. Sensitivity for urine was 1 μg/L. The intra-assay CV was 2.2% at a MBOCA concentration of 100 μg/L. We believe that this method is acceptable for routine measurement of MBOCA in urine from individuals exposed to this industrial chemical.

Additional Keyphrases: occupational hazards · toxicology

Methylenebis(2-chloroaniline) (MBOCA) is widely used in industry as a curing agent for urethane polymers and epoxy resins (1). Since 1980, all the MBOCA used in the United States has been imported from Japan at an estimated rate of 450 000 to 1 600 000 kg per year (2). MBOCA has been regarded as an occupational carcinogen, because evidence of carcinogenesis by MBOCA has been reported in animal

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experiments (3, 4). Thus, levels of exposure to MBOCA in workers should be strictly controlled. The major routes of absorption of MBOCA are inhalation and skin (1). However, the exposure level cannot be accurately estimated only by environmental monitoring because, in addition to inhalation, considerable MBOCA is absorbed through the skin (1). Thus, biological monitoring is essential to assess exposure to MBOCA in workers—e.g., by measuring urinary MBOCA by gas chromatography with electron capture detection (GC-ECD) (5) or by HPLC with spectrophotometry (6).

Here we report our development of a highly sensitive and selective method—HPLC with electrochemical detection (HPLC-ECD)—for determining MBOCA in urine. We also describe a simple, selective method for sample preparation, ion-paired solid-phase extraction, to facilitate application of this method to routine biological monitoring of urinary MBOCA.

Materials and Methods

Reagents. MBOCA was purchased from Tokyo Kasei Co., Tokyo, Japan. 1-Heptanesulfonic acid, sodium salt, was obtained from Aldrich Chemical Co., Milwaukee, WI. Potassium dihydrogen phosphate dihydrate, "HPLC grade" methanol, and acetic acid were purchased from Katayama Chemicals Co., Osaka, Japan. Other chemicals used were of the highest quality available. The water used was de-ionized and purified with a "Puric-R" system (Organo, Tokyo, Japan). Mobile phases for HPLC were filtered through a membrane (0.45-μm pore size, 47 mm i.d.; Millipore Filter Corp., Bedford, MA) before use.

Sample collection. Urine samples from nonexposed controls and from workers exposed to MBOCA were collected at the end of the 8-h workshift into polystyrene disposable tubes and stored at -20 °C until analysis.

Standard solution. After recrystallizing MBOCA three times from aqueous methanol, we dissolved 100 mg of it in 100 mL of methanol containing 10 mL of acetic acid per liter; it was stored at 4 °C, and used as a stock standard. We prepared working standards (1-500 μg/L) every day by diluting the stock standard with water.

Preparation of samples. We mixed 8 mL of urine samples or standard solutions with 2 mL of methanol containing 50 mL of acetic acid and 5 g of heptanesulfonate per liter, and centrifuged the mixture for 10 min at 3000 × g. Supernatant fluids (9.5 mL) were loaded onto disposable ODS columns (Baker-10 SPE system, Model 7020; J. T. Baker Chemical Co., Phillipsburg, NJ), which had been previously prepared by slow passage of 3 mL of methanol, followed by 3 mL of extracting solution (per liter: 200 mL methanol, 10 mL acetic acid, and 1 g heptanesulfonate). We then dried the columns with a stream of air and washed them with 1 mL of the extracting solution, followed by 2 mL of water, and then dried them again. MBOCA was eluted from the column with three 0.5-mL portions of methanol. Before HPLC, we diluted the eluate to 3 mL total volume with water. Before use, all glassware was washed with dilute (0.1 mol/L) hydrochloric acid to remove residual amines.

Chromatographic procedure. Inject 20 μL of the extracted samples onto a 150 × 4 mm (i.d.) column of octadecyl silica (TSK-GEL, Model ODS 80-Tm 5-μm particles; Toso Co., Tokyo, Japan) with an autosampler (Model 638–08; Hitachi Corp., Tokyo, Japan). We used a stepwise gradient elution with a liquid chromatograph (Model 638–30; Hitachi Corp.) at a flow rate of 1.0 mL/min.

Elute the column with mobile phase A (630 mL of methanol per liter) for 14 min, then wash with mobile phase B (850 mL of methanol per liter) for 2 min and then equilibrate with mobile phase A for 6 min. Another sample can be injected every 22 min. Mix the column eluate with potassium dihydrogen phosphate buffer (0.1 mol/L) at a flow rate of 1.0 mL/min, with a constant-flow pump (Model CCPD; Toso Co.), then lead the mixture into a mixing coil (10 m × 0.4 mm, i.d.), and introduce the eluate into an amperometric detector (Model E-502 detector with a glassy carbon electrode; Iriki Co., Kyoto, Japan) operated at 0.80 V potential between the working electrode and the Ag/AgCl reference electrode. Calculate the concentration of MBOCA from the peak area with a data processor (e.g., Model CR-2A; Shimadzu Corp., Kyoto, Japan). Determine the standard solution after each 10 urine samples. All separations are conducted at room temperature.

Results

A typical chromatogram of a standard mixture (45 μg/L) is shown in Figure 1. MBOCA was eluted at 12.4 min. Figure 2 depicts the relationship between applied voltage and peak area. MBOCA was not detectable below 0.4 V. The peak area increased with applied voltage and reached maximum at 0.80 V. The peak area increased linearly with the concentration of MBOCA over the range of 1-500 μg/L (r = 0.997).

The intra-assay CV was 2.2% (n = 10) and the inter-assay CV was 5.6% (n = 3) for the 100 μg/L standard solution.
Figure 3 shows representative chromatograms of urine samples. No peak was observed at 12.4 min in the urine from a control subject (A) and the added MBOCA peak was clearly separated from other peaks (B). Determination of urine samples could be repeated every 21 min. Sample recoveries were 99.5 (SD 3.3%, n = 5) and 97.1 (SD 4.5, n = 5) for 100 and 20 µg/L of added standard, respectively. Figure 3C shows a chromatogram of a urine sample obtained from an individual exposed to MBOCA for 8 h. MBOCA appeared at 12.4 min, separated from other peaks. The mean concentration of MBOCA in the urine at the end of the 8-h work shift was 66 µg/L (range 17–97 µg/L, n = 3). No interference of the MBOCA peak by imipramine was noted (data not shown), although interference has been reported for the GC-ECD method (7). The detection limit for this method was 1 µg/L in urine (signal/noise ratio = 5).

Discussion

Carcinogenicity of MBOCA has been reported from animal studies. Urinary bladder cancer was observed in dogs given 8–15 mg per kilogram body weight per day, orally (3). The incidence of hepatomas was significantly increased in female mice administered MBOCA 1000 mg/kg body weight per day, orally (4). Measurement of MBOCA in urine is considered to be the best detection method because MBOCA absorption through the skin is not negligible (1). The California Occupational Safety and Health Administration recommends that the concentration of MBOCA in urine should not exceed 100 µg/L (8).

Several methods have been used to determine MBOCA in urine. One is gas-chromatography with electron capture detection (GC-ECD), which was adopted by the National Institute for Occupational Safety and Health as a standard method (5, 7). The determination limit for urine for this method is 1 µg/L. HPLC with spectrophotometry (6) can be used to determine urinary MBOCA after 100-fold concentration of the samples (determination limit, 10 µg/L in urine). A very sensitive HPLC-ECD method (determination limit, 2 µg/L in extracted solution) has been reported for measuring MBOCA in air (9). However, without selective sample preparation it is not suitable for measuring urinary MBOCA, because the other substances such as amines and phenols in urine may cause interference (Figure 3D).

Recently, solid-phase extraction methods involving disposable columns were used for sample purifications of various kinds of biological materials (10). We examined a sample-preparation method involving a disposable ODS column, but the MBOCA was insufficiently purified and the interference peaks could not be efficiently removed (Figure 3D). To make the extraction procedure more selective, we adopted an ion-paired solid-phase extraction method involving use of a single ODS column. Using heptanesulfonate as a paired ion, we could extract MBOCA in 200 mL/L methanolic solution onto the ODS column. After washing out excess paired ion with water, we could then elute MBOCA selectively with methanol. As shown in Figure 3A, B, and C, interference peaks were exclusively eliminated and good analytical recoveries were obtained.

Because high conductivity of the mobile phase is indispensible for electrochemical detection, the mobile phase should contain an electrolyte such as potassium dihydrogen phosphate. However, this substance is not very soluble in the methanol/water (60/40 by vol) solution used in separating MBOCA. Thus, we developed the HPLC system by mixing the eluate of the analytical column with the phosphate buffer after separating MBOCA. The period for cleaning the analytical column with mobile phase B (Figure 1) could then be inserted into the flow program.

The new method reported here offers simple, selective sample preparation and automatic execution of HPLC analysis. With an autosampler, more than 70 samples can be determined in a day.

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Automating the Quantification of Heme in Feces

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We present a modification of the HemoQuant assay, a good but lengthy and tedious method for determining heme in feces by means of its transformation to porphyrins. The laborious extraction procedure was replaced by a simple centrifugation procedure. The nonhomogeneous hot oxalic acid suspension was replaced by acetic acid. We observed no significant difference in results between samples analyzed by the older method vs the present modification (r = 0.996, n = 52). Mean (and SD) analytical recoveries of added hemoglobin and protoporphyrin were 99% (7%) and 93% (6%), respectively. The analytical procedure can now be automated by using discrete samplers and a flow-through fluorometer. Initial sampling and dilution of feces are still done manually, however. The excellent specificity, sensitivity, and overall analytical performance of the original method are retained, while circumventing the practical inconveniences of this reliable screening test for occult blood in feces.

Additional Keyphrases: hemoglobin, occult blood, protoporphyrin

Because, clinically, tests for heme in feces are used early in forming a diagnosis, such a test should be specific, relatively simple, and suitable for automation, so that large numbers of samples can be analyzed. The method of Schwartz et al. (1) produces reliable and quantitative data for heme in feces. It is superior to any of the methods based on the leuko-dye principle, but it is cumbersome and time consuming, which precludes its use as a routine procedure.

Previously, we (2) reported some modifications for simplifying the method. Here we describe further developments of the method that permit automation of the reaction process. The main obstacles to automation of the test procedure as originally described are the extraction procedures and the use of a hot, nonhomogeneous suspension, which cannot be transferred by automatic devices. In our earlier adaptation (2) we omitted extracting the fecal homogenate with organic solvents, as reiterated briefly here. In addition, we now have modified the procedure and the reagents used for converting the heme to (pro)porphyrin, using glacial acetic acid, ferrous ions, and HCl to reduce hemin and release Fe from the porphyrin skeleton (3, 4). We also examined incubation time and temperature, composition of blank assays, the arrangement of standards, and the possibility of combining various reagents into a single reagent.

Materials and Methods

Hemoglobin standards. Hemoglobin (Hb) stock standard was made by dissolving 595 mg of Hb in 50 mL of water to give a heme concentration of 700 μmol/L. For a series of standards, volumes containing 35–210 nmol of heme were freeze-dried and kept at −20 °C. Before use, we reconstituted a series of standards on the day of the determination. Because Hb standards in water were unstable in the assay, we used fecal water as the medium for reconstituting these standards. Fecal water was prepared by centrifuging (10 min, 1500 × g) an emulsion of normal feces in water, 1 g of feces in 30 mL of water, and using the supernate. Normal fecal material can be selected from prior specimens or by analyzing feces collected for this purpose, with appropriate addition of freshly made heme standards in water. We selected material that yielded a reading for the sample that was comparable to its blank reading. The contribution to the reading for the heme standards is then acceptably low.

Protoporphyrin standards. Standards of protoporphyrin (PP) to be used for analytical recovery studies were prepared as described earlier (2). We freeze-dried PP standards, 10–60 μmol/L, and kept them at −20 °C. On the day of the assay we reconstituted them with fecal water, as described for Hb standards.

Sample preparation. Fecal material (preferably a 24-h collection) was weighed and diluted with twice its weight of water and mixed with a domestic "milk-shake" blender. From this mixture we transferred duplicate 1-g samples to 12-mL polystyrene tubes. We also filled one tube with 3.0 g of the emulsion, to use in measuring fecal dry weight (by freeze-drying or any other suitable technique). To the 1.00-g fecal samples we added 5.0 mL of a 5:1 (by volume) mixture of 2-propanol and 1 mol/L HCl. We mixed the contents of each tube, centrifuged for 10 min at 1500 × g, and transferred the supernates to polystyrene tubes. We assessed analytical recovery by assaying a mixture of equal volumes of sample (prepared by this same pretreatment scheme) and of one of the standards (PP or Hb, each dissolved in fecal water).

Assay procedure. To 50 μL of the supernates from samples, standards, and recovery mixtures, add 1.0 mL of glacial acetic acid, mix, then add 50 μL of a freshly prepared aqueous solution of FeSO₄·7H₂O (0.12 mol/L) and HCl (4.5 mol/L). Mix well, then, without delay, incubate all samples for 30 min at 60 °C. Next add 2.0 mL of an equivalence

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