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Short communication

Development of an analytical method to confirm toxic trimethylated tin in human urine

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ABSTRACT

Dimethyltin dichloride (DMTC) is widely used as a heat stabilizer in manufacturing the polyvinyl chloride. We previously reported a case of acute DMTC poisoning with neurological manifestations very similar to trimethylated tin (TMT) encephalopathy, based on results of speciation analysis of methylated tins in the patient's urine with use of a combination of high performance liquid chromatography and inductively coupled plasma–mass spectrometry (HPLC–ICP–MS), which yielded peaks corresponding to DMT and TMT. In this study, we developed an analytical method to confirm TMT in urine using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), and found TMT molecular ion in the patient's urine.

1. Introduction

Dimethyltin dichloride (DMTC) is widely used as a heat stabilizer in rigid polyvinylchloride resins [1,2]. Recently, DMTC has been used to produce transparent conductive films for liquid crystal panels [2]. Among the methylated tins, trimethylated tin (TMT) is the most toxic, and its marked neurotoxicity in humans has been demonstrated in cases of accidental exposures [3–6]. In animal experiments, however, the toxicity of dimethylated tin (DMT) differs from that of TMT [7–10]. We previously reported a case of acute poisoning due to DMTC exposure [11]. Since the clinical signs in this patient were the severe neurological manifestations very similar to those of TMT encephalopathy, the methylation of DMT to TMT was suspected. We then developed a method of speciation analysis of methylated tins, such as monomethylated tin (MMT), DMT, and TMT, using a combination of high performance liquid chromatography and inductively coupled plasma–mass spectrometry (HPLC–ICP–MS), and chromatographic peaks corresponding to each DMTC and trimethyltin chloride (TMTC) were detected in the patient's urine, whereas peaks for methyltin trichloride (MMTC) and inorganic tin were not [11].

Previous *in vitro* and *in vivo* experimental studies have shown that organotin compounds undergo biotransformation by dealkylation [12]. No methylation of such compounds has been reported in animal experiments. If methylation of DMT to TMT occurs to a significant extent in humans, the severe neurotoxicity of DMT in the patient can be explained. In this study, we developed an analytical method to confirm TMT using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS).

2. Experimental

2.1. Chemicals

Methyltin trichloride (MMTC), dimethyltin dichloride (DMTC), trimethyltin chloride (TMTC), methanol (HPLC grade), formic acid (99% approximately, LC–MS grade), and hydrochloric acid (35–37%, analytical grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ammonia solution (25.0–27.9%, atomic absorption grade) and germanium standard solution were purchased from Kanto Chemical Co. (Tokyo, Japan). Ammonium formate (99.995+%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Tap water was purified through Milli-Q Element A10 (Millipore Japan, Tokyo, Japan) and used as ultra-pure water.

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2.2. Urine samples

The urine samples examined in the present study originated from a patient, who had been exposed to DMTC for 4 days in a workplace and was then immediately transferred to a hospital on the 4th day of exposure. Urine samples were collected on the 6th, 9th, 11th, 14th, 17th, 19th, and 24th days of hospitalization [11].

2.3. Separation and detection of dimethylated tin and trimethylated tin by HPLC–ICP–MS

Ten-microliter portions of 5-fold-diluted urine samples were injected into an HPLC system (HP1100, Agilent, CA, USA). DMT and TMT were separated using a reversed phase polymer column, Shodex Asahipak ODP-50 4D (150 × 4.6 mm i.d., Shoko Co., Tokyo, Japan), under the following conditions: mobile phase of 10 mM ammonium formate containing 0.1% formic acid; flow rate, 0.8 mL/min; column temperature, 40 °C. TMT was eluted at 4.9 min. An ICP–MS (HP4500, Agilent, CA, USA) was used for detection of tin isotopes of m/z 116 ($^{116}Sn^+$), m/z 118 ($^{118}Sn^+$), and m/z 120 ($^{120}Sn^+$).

2.4. Clean up of urine samples for HPLC–MS/MS analysis

Solid-phase extraction (SPE) was applied to clean urine samples using an Oasis MCX 1cc cation exchange cartridge (Waters, MA, USA). According to the manufacturer's standard protocol, the cartridge was conditioned and equilibrated with 1 mL of methanol and ultra-pure water in sequence. After loading 2 mL of supernatant of urine samples centrifuged at 12,000 × g for 10 min onto the cartridge, it was washed with 1 mL of 2% aqueous formic acid and 1 mL of methanol. TMT was then eluted with 1 mL of ammonia solution:methanol (5:95) and directly collected into a polypropylene test tube containing 1 mL of 0.1 M hydrochloric acid. In order to calculate the recovery of TMT on this SPE, the eluate was measured by HPLC–ICP–MS with post-column introduction of 0.1 mg/L of germanium solution as internal standard for ICP–MS. The peak area ratios of Sn (m/z 118) to Ge (m/z 72) were used for calculation. Recovery was determined by duplicate measurements of standard solutions containing each 0.1 and 1.0 mg/L each of TMTC.

2.5. Confirmation of TMT in urine samples by HPLC–MS/MS

Ten-microliter portions of urine samples pretreated with the SPE was injected into an HPLC system (Alliance 2695, Waters, USA) connected to a Shodex Asahipak ODP-50 2D reversed-phase polymer column (150 × 2.0 mm i.d., Shoko Co., Tokyo, Japan) under the following conditions: mobile phase of 10 mM ammonium formate containing 0.1% formic acid; flow rate, 0.2 mL/min; column temperature, 40 °C. TMT was eluted at 3.6 min. Detection of TMT was performed by a Quattro micro API tandem mass spectrometer (Waters, USA) with electrospray ionization (ESI) positive ion mode by setting capillary voltage to 0.5 kV, ion source temperature to 120 °C, desolvation nitrogen gas temperature to 400 °C, desolvation gas flow to 600 L/h, and cone gas flow to 50 L/h. Collision-induced dissociation (CID) was performed with argon gas introduced into the collision cell placed between the quadrupoles. Multiple reaction monitoring (MRM) was optimized to detect CID fragmentations of TMT as shown in Table 1. The divert valve was set to introduce only the HPLC effluent to the mass analyzer from 2 to 5 min.

Table 1
Parameters of multiple reaction monitoring (MRM) for TMT

Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
165.0	134.9	25	20
165.0	150.0	25	15
183.1	134.9	30	25
183.1	150.0	30	20

3. Results and discussion

One aim of the present study was to confirm with use of HPLC–MS/MS that the chromatographic peaks previously detected by HPLC–ICP–MS are DMT and TMT. In a method previously developed for speciation analysis of methylated tins, a cation exchange column (Shodex NN-614, Shoko Co., Tokyo, Japan) with the mobile phase consisting of 5 mM nitric acid, 6 mM ammonium nitrate, and 1.5 mM 2,6-pyridinedicarboxylic acid was used [11]. However, this eluent produces a non-volatile substance in the ESI area that interferes with MS detection [13]. In this study, ESI-compatible chromatographic conditions capable of yielding quality mass spectra and chromatographic separation of methylated tins were investigated using aqueous solutions of them. Since reversed-phase conditions and a volatile mobile phase are generally preferred for ESI–MS, a mixture of formic acid with ammonium formate was selected as a mobile phase. In addition, we observed irreversible adsorption of methylated tin compounds to octadecylsilylated (ODS) silica gel, which is commonly used as a stationary phase. In order to minimize this irreversible adsorption, an octadecylated polyvinyl alcohol-based column, of the Shodex Asahipak ODP-50 series (Shoko Co., Tokyo, Japan), was selected. Although this column yielded single chromatographic peaks of TMT and DMT, MMT still exhibited undesirable chromatographic behaviors presumably due to irreversible adsorption. This chromatographic condition was thus not suitable for analysis of MMT. The HPLC–ICP–MS chromatograms of DMT and TMT in both aqueous solution and the patient's urine samples are shown in Fig. 1. TMT was clearly detected in all of 7 measured urine samples of the patient exposed to DMTC at the same retention time (4.9 min) as TMTC, whereas peaks of DMT were broadened around 12–15 min under these chromatographic conditions compared with the sharp peak obtained with use of a cation exchange column [11].

For HPLC–ICP–MS measurement, urine samples were simply pretreated by 5-fold dilution with ultra-pure water, and tin compounds were detected. However, in none of the diluted urine samples were DMT and TMT detected by HPLC–MS/MS equipped with ESI ion source, presumably due to suppression of ionization by interfering urine matrix components. Furthermore, dilution of urine samples was limited by insufficient sensitivity of this method of MS/MS detection. Thereafter, additional pretreatment of urine

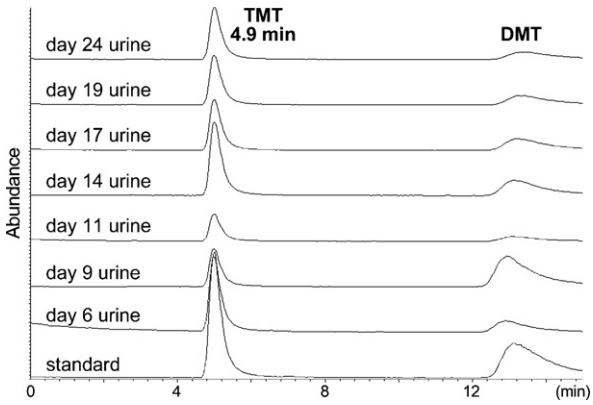

Fig. 1. HPLC–ICP–MS chromatograms. HPLC–ICP–MS chromatograms recorded at m/z 118 (corresponding to $^{118}Sn^+$) for 0.1 mg Sn/L aqueous solution of dimethyltin dichloride (DMTC) and trimethyltin chloride (TMTC), and for patient's urine samples collected on hospital days 6, 9, 11, 14, 17, 19, and 24 are shown. The ordinates are normalized to the same abundance.

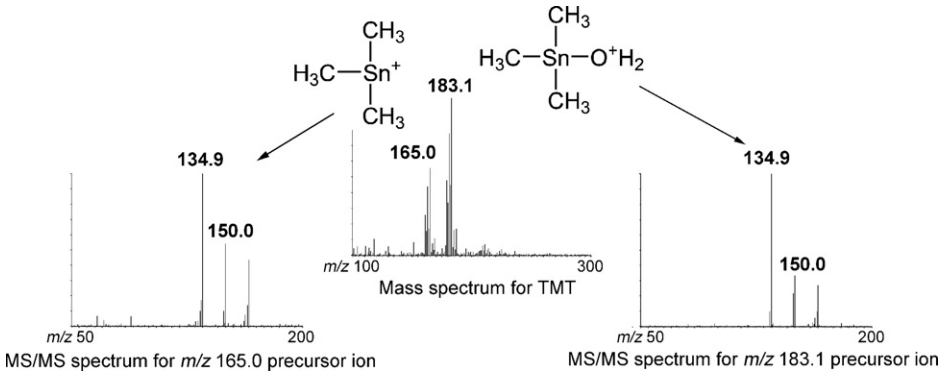

Fig. 2. Mass spectrum and molecular ion structure (center), and MS/MS spectra (left and right) of TMT in aqueous solution. Details are given in Section 3.

samples was necessary with recent SPE to remove interference. Since TMT had been found to have good retention characteristics in cation exchange stationary phase [11], an Oasis MCX cation exchange cartridge (Waters, USA) was examined for recovery of TMT from urine samples. Blank (TMT free) urine samples were spiked with TMTC and were fractionated through the Oasis MCX cartridge. TMT was detected as nearly the only fraction eluted with a mixture of ammonia solution:methanol (5:95). In order to evaluate the rate of recovery of TMT, triplicate blank (TMT free) urine samples each spiked with TMTC as 1.0 mg Sn/L were pretreated with this SPE. The calculated recovery of TMT was 98 ± 1% (n = 3). Thereafter, this SPE clean-up was applied to the patient's urine samples, which contained 0.11–0.30 mg Sn/L of TMT.

In order to detect TMT cations with high sensitivity in the HPLC–MS/MS system, multiple reaction monitoring (MRM) mode, which can selectively detect a product ion of interest from a specified precursor ion, was used. This mode was especially useful for detecting known analytes of interest. The specific pairs of precursor and product ions shown in Table 1 were established using TMTC aqueous solution as follows. The mass spectrum of TMTC (Fig. 2, center) exhibited two major ions such as m/z 165.0 and m/z 183.1, which correspond to monovalent trimethylated tin cation ($[SnMe_3]^+$) and its hydrated ion ($[SnMe_3 + H_2O]^+$), respectively. As shown in Fig. 2, the MS/MS spectra for these two precursor ions exhibited two intense fragment ions of m/z 134.9 ($[SnMe]^+$) and m/z 150.0 ($[SnMe_2]^+$), which indicate losses of methyl groups from a trimethylated tin cation by the CID. Those four ion pairs of m/z 165.0 and 134.9, m/z 165.0 and 150.0, m/z 183.1 and 134.9, and m/z 183.1 and 150.0 were chosen for identification of TMT. Fig. 3 shows that the MRM chromatograms of 7 urine samples from the patient and a blank urine sample spiked with TMTC as 1.0 mg Sn/L (top) exhibit a specific peak of TMT at 3.6 min. These findings demonstrated the presence of TMT cation in 7 urine samples of the patient, and indicate that the present analytical method is highly useful and effective in confirming TMT cation in urine.

Since the S/N ratios (peak-to-peak) were found 35–60 (depending on the precursor ions, data not shown) for TMTC aqueous solution of 1 mg Sn/L, the limit of detection (S/N = 3) for this MRM measurements is expected to be 0.1 mg Sn/L or less. This value would be appropriate, since 0.11–0.30 mg Sn/L of TMT was actually contained in the 7 patient's urine samples found to contain TMT, as seen in Fig. 3.

Inorganic arsenic is methylated to dimethylarsinic acid (DMA) in humans, and this reaction may have the potential for the detoxification of arsenic, though several animal species such as marmoset monkeys and chimpanzees lack the ability to methylate arsenic [14]. In addition, biotransformed compounds of this type and their

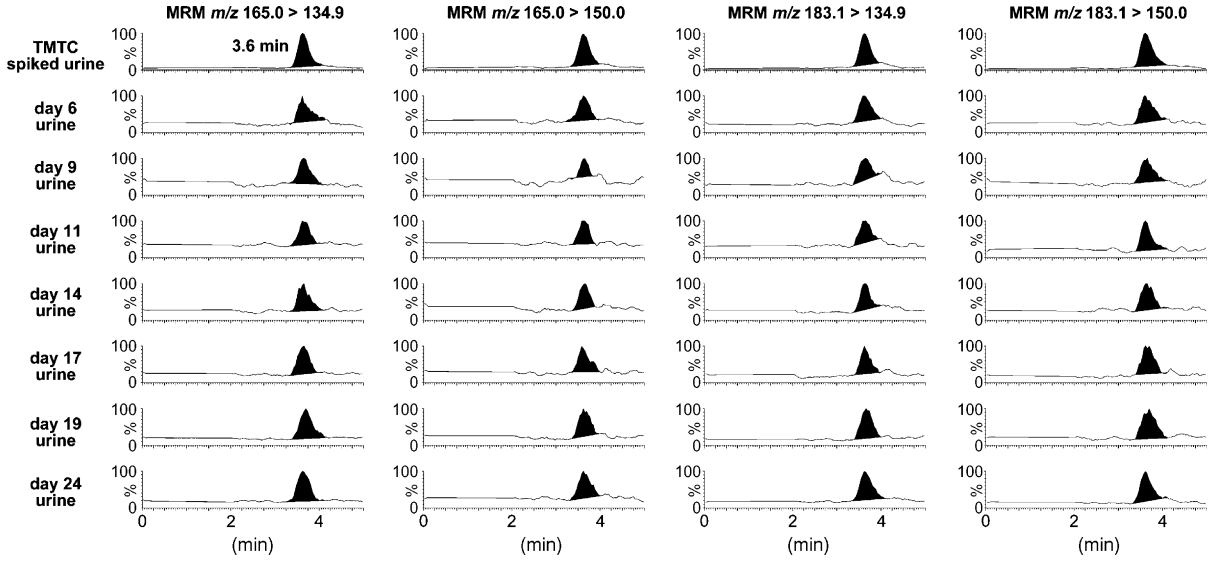

Fig. 3. Multiple reaction monitoring (MRM) chromatograms for blank urine spiked with TMTC and patient's urine samples. The two chromatograms at left show the product ions, m/z 134.9 and 150.0, derived from a precursor ion of m/z 165.0, while the other two chromatograms at right show the product ions derived from a precursor ion of m/z 183.1. The ordinates are normalized to 100% for the peak of each MRM chromatogram.

rates of formation are reported to differ among animal species [15]. We recently detected methylation of DMT to TMT in rats and mice administered DMTC [16], though the rates of conversion were not high (less than 1%) in both species. If the methylation of DMT to TMT occurred in humans in a fashion similar to that in these rodents, the severe neurological manifestation in patients exposed to DMTC in the work environment can be explained, though TMTC as a by-product (less than 0.3%) existed in industrial DMTC [11].

Since use of DMTC has recently extended to the advanced electronics industries [2], care in handling of DMTC is required. Our analytical method to confirm TMT in urine samples will be useful for elucidating of the mechanism of toxic biotransformation.

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