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Determination of nerve agent metabolites in human urine by femtosecond laser ionization mass spectrometry using 2-(bromomethyl)naphthalene as a derivatizing reagent

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ABSTRACT

Nerve agent metabolites (NAMs) derived from alkyl methyl phosphonic acids, such as ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), and pinacolyl methylphosphonic acid (PMPA), were extracted from human urine using diethyl ether as an extractant. After exchanging the diethyl ether solvent to acetonitrile, the analytes were derivatized with 2-(bromomethyl)naphthalene (BMN). The reaction products of the BMN and NAMs, i.e., MN-EMPA, MN-IMPA, and MN-PMPA, were separated by gas chromatography (GC) and measured by mass spectrometry (MS) using a femtosecond laser emitting at 267 nm as the ionization source for resonance-enhanced two-photon ionization (RE2PI). The limits of detection (LOD) were <1 ng/mL for these analytes. The use of BMN increased the volatility of the analytes for separation by GC and also increased the ionization efficiency via the RE2PI process as the result of presence of a naphthalene functional group. A two-dimensional GC-MS display can be used for comprehensive analysis of NAMs, by-products, and impurities in the sample. Then, this approach could be used to confirm the use of chemical weapons and for forensic identification.

Keywords:

Nerve agent metabolites

(Bromomethyl)naphthalene

Gas chromatography

Femtosecond laser ionization

Mass spectrometry

1. Introduction

Organophosphorus-based nerve agents (OPAs) were developed prior to the start of World War II and, since then, have been used as chemical warfare agents (CWAs). They are very toxic and harmful to humans [1,2]. The Chemical Weapon Convention has listed several compounds including sarin, soman, and VX as nerve agents (NAs) [3]. Recent terrorist attacks have caused significant tensions to citizens in several countries and serious problems related to regional security. For example, the subway attack with sarin that occurred on 20 March 1995, in Tokyo, Japan, killed 12 persons and severely injured 50 more (some of whom died later) and caused temporary vision problems for nearly 1000 persons [4]. CWAs act very rapidly on humans and are easily decomposed to different compounds in the human body. In fact, OPA is excreted as metabolites in the urine within the first 24-72 h after exposure [5-7]. For example, sarin, soman, and VX are decomposed to nerve agent metabolites (NAMs), i.e., ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), and pinacolyl methylphosphonic acid (PMPA), respectively. The alkyl groups in these compounds subsequently decompose to produce methyl phosphonic acid (MPA) within two weeks. Therefore, the use of a NA in a terrorist attack has been confirmed by measuring NAM levels in a body fluid such as urine. Thus, the time of occurrence of a terrorist attack can be estimated from the concentration ratio of NAMs. Moreover, information concerning the side-reaction products of NA and impurities contained in the sample can be used in forensic identification, preventing future terrorist attacks. Therefore, it would be desirable to develop a sensitive and selective analytical method for the “comprehensive analysis” of NAMs in human urine samples.

A combination of mass spectrometry (MS) and gas chromatography (GC) or liquid chromatography (LC) can be used in NAM measurements because of their high sensitivity and

selectivity [8-13]. It should be noted that NAM are phosphonic acids and they are nonvolatile in nature, making the direct analysis by GC-MS a challenging task. The approach of using LC-MS combined with electrospray ionization (ESI/MS) is straightforward and is useful for the measurement of NAMs without a derivatization reaction [8]. This method, however, suffers from limited performance in separation by LC (it is suggested using GC-MS for identification of NAMs before analysis by LC-MS). On the other hand, excellent separations can be achieved using GC-MS and the method has been widely used for this purpose. However, it is necessary to use a derivatization technique to increase the volatility of the compound. For example, *tert*-butyldimethylsilyl chloride (TBDMSC) and 2,3,4,5,6-pentafluorobenzyl bromide (PFBBBr) have been employed as the derivatization reagents [14]. There are several ionization techniques available such as electron ionization (EI) and chemical ionization (CI). In general, extensive fragmentation occurs when EI is used because of the large excess energy in the process of ionization, which often prevents the observation of a molecular ion. In addition, NAMs have similar molecular structures and provide similar fragment patterns, which makes a reliable analysis of NAMs difficult. On the other hand, CI provides no or substantially less fragment ions, making fingerprinting identification from the fragment pattern difficult. For this reason, NAMs are often derivatized with PFBBBr, i.e., PFB-NAMs, which are frequently ionized by positive-ion or negative-ion CI (PICI or NICI) and the resulting positively- or negatively-charged ion is then decomposed and measured by EI/MS. This technique, referred to as MS-MS, is useful for the selective and sensitive analysis of NAMs [10-12]. However, this technique can be employed for the detection of components that are specified prior to the measurement, thus making the determinations of various NAMs and unknown side-reaction products/impurities in the sample difficult. In order to solve this problem, it is suggested developing a new MS technique that permits the “comprehensive analysis” of constituents with similar properties

and provides a molecular ion and fragment ions as well.

Photoionization mass spectrometry is a technique that permits the simultaneous observation of both molecular and fragment ions. Femtosecond ionization mass spectrometry (FI/MS) is particularly interesting in the respect, since sub-femtogram detection limits for polycyclic aromatic hydrocarbons have been reported [15]. It is also noteworthy to mention that it has been utilized for the determination of persistent organic pollutants (POPs) such as dioxins and pesticides [16], carcinogenic compounds such as nitro polycyclic aromatic hydrocarbons [17], allergy substances in a fragrance [18], and explosives such as trinitrotoluene and triacetone triperoxide [19]. It should also be noted that, in most cases, a molecular ion can be observed through resonance-enhanced multiphoton ionization (REMPI), even for molecules with very short excited-state lifetimes, when an ultraviolet (UV) femtosecond laser is used as the ionization source. In fact, it has been previously reported that NAMs can be measured using PFBBr as a derivatization reagent [20]. In that study, the ionization efficiency was relatively poor due to the presence of the chromophore of the PFB group in PFB-NAM, since it has no absorption band at the wavelength of the third harmonic emission of a Ti:sapphire laser (267 nm) and is ionized through a process of non-resonant two-photon ionization (NR2PI). It is well known that there are many organic molecules that can be efficiently ionized at 267 nm. For example, naphthalene has an ionization energy of 8.144 eV and can be measured efficiently by resonance-enhanced two-photon ionization (RE2PI) at a wavelength of 267 nm [21]. Therefore, using a derivatizing agent that contained such a group, it would be possible to sensitively determine NAM using the above approach.

In this study, we report on the determination of EMPA, IMPA, and PMPA in a human urine sample by derivatizing them with 2-(bromomethyl)naphthalene (BMN) that contains a naphthalene chromophore for efficient RE2PI and a 2-bromomethyl group for combination with an -OH group in

NAMs. The derivatized compounds of MN-NAMs were separated by GC and measured by a time-of-flight mass spectrometer (TOF-MS) for comprehensive analysis using a UV femtosecond laser emitting at 267nm for efficient RE2PI.

2. Experimental section

2.1. Apparatus

Figure 1 shows a diagram of the experimental apparatus used in this study, which is a combination of a commercial GC (6890N, Agilent Technologies, Santa Clara, CA, USA) and an MS developed in our laboratory (HG-1, Hikari Giken, Fukuoka, Japan). The third harmonic emission (267 nm, 0.1 mJ) of a Ti:sapphire laser (800 nm, 100 fs, 1 kHz, 1 mJ, Libra, Coherent, Santa Clara, CA, USA) was used as the ionization source. The analytes were separated using a DB-5ms column (30 m in length, 0.25 mm inner diameter, 0.25 μ m film thickness). The temperature program of the GC oven was as follows; initial temperature 50 °C held for 1 min, a rate of 10 °C/min to 80 °C held for 1 min, then 15 °C/min to 280 °C held for 5 min. The temperatures of the GC inlet port and of the transfer line between the GC and MS were maintained constant at 250 and 280 °C, respectively. Helium was used as the carrier gas, and the flow rate was 1 mL/min. The amount of sample injected into the GC was 1 μ L. The analytes in the molecular beam were ionized by focusing the UV femtosecond laser beam. The resulting induced ions were accelerated toward a flight tube. An assembly of microchannel plates (F4655-11, Hamamatsu Photonics, Shizuoka, Japan) was used to detect the ions. The signal was recorded by a digitizer (Acqiris AP240, Agilent Technologies), and the data were analyzed and used for the construction of a two-dimensional display of GC-MS using a home-made software programmed by Visual Basic. The GC combined with a flame ionization detector (FID) was used to

determine the optimum conditions for derivatizing the NAMs with BMN.

2.2. Extraction

The solvent extraction procedure used in this study is summarized in Figs. 2 (A) – (E). The sample was prepared by dissolving 100 ng of EMPA, IMPA, and PMPA (they are nontoxic and commercially available from Sigma - Aldrich Japan, Tokyo, Japan) in 1 mL of human urine (derived from one of the authors, Vu Van Son) in a vessel made of glass (1.5 mL) (A). Non-polar interferences in the urine were removed by extracting the sample with 1 mL of n-hexane (Wako Pure Chemical Industries, Tokyo, Japan) (B). The pH value of the sample solution was adjusted at 1.8~2 by adding 8~10 μ L of 36% hydrochloric acid (C). Diethyl ether (1 mL) (Wako Pure Chemical Industries) was then added to the sample solution containing the NAMs for solvent extraction. The sample solution was manually shaken several min and then allowed to stand at room temperature for 2 h. The organic phase was then removed by a pipette and was transferred to another vessel (D). To remove water, 5 mg of anhydrous Na_2SO_4 was added to the solution and the vessel was shaken 10 min by hand, and then the supernatant was transferred by a pipette to another vessel (E). The sample solution was heated to 35 °C to remove the solvent for the subsequent derivatization reaction.

2.3 Derivatization

The NAMs (EMPA, IMPA, and PMPA) in the vessel were dissolved using a solvent mixture of 699 μ L of acetonitrile (Wako Pure Chemical Industries) and 1 μ L of a stock solution (0.4 mg/mL, 1.8 μ M/mL) of BMN (Sigma - Aldrich Japan) dissolved in acetonitrile was added to the sample solution (totally 700 μ L). A 300- μ L portion of N,N-dimethylformamide (DMF, Wako Pure Chemical Industries), a polar aprotic solvent, was added to partly dissolve 0.1 mg of potassium carbonate

(K₂CO₃, Wako Pure Chemical Industries) which serves as a catalyst/base to dissociate a proton from OH in phosphonic acid to react with BMN. The 1-mL solution was then mixed using a magnetic stirrer at a specified temperature (45~70 °C) for several hours (1~15 h). This solution containing MN-EMPA, MN-IMPA, and MN-PMPA in the solvent mixture of acetonitrile and DMF was used as the sample and was injected into the GC.

2.4. Quantum chemical calculations

The spectral properties of MN-EMPA, MN-IMPA, and MN-PMPA were predicted based on quantum chemical calculations. The optimized geometry of the ground and excited states and the harmonic frequency were calculated using the B3LYP method [22], based on density functional theory (DFT) with a cc-pVDZ basis set [23]. Time-dependent DFT (TD-DFT) was used to calculate the lowest 40 singlet transition energies and their oscillator strengths at the levels of B3LYP/cc-PVDZ and B3LYP/cc-PVTZ [24,25]. The absorption spectra were obtained by assuming a Gaussian profile with a half width at half maximum of 0.333 eV for each transition. The vertical ionization energy (*IE*) and its half value (*IE*/2) were also calculated from the difference between the energies of the ground and ionic states.

3. Results and discussion

3.1. Spectral properties of NM-NAM

Figure 3 shows the absorption spectra and calculated spectral properties of the MN-NAMs. These compounds are ionized via the B-band of naphthalene located at around 300 nm. The ionization energy of MN-EMPA, MN-IMPA, and MN-PMPA were calculated to be 7.764, 7.770, and 7.768 eV,

respectively, using B3LYP/cc-PVDZ (7.839, 7.850, and 7.851 eV, respectively, using B3LYP/cc-PVTZ). Since a UV femtosecond laser (267 nm, 4.652 eV) was used as the ionization source, the derivatized compounds were ionized through a two-photon process, i.e., RE2PI ($4.652 \text{ eV} > IE/2$). The excess energy in the ionization process was calculated to be 1.537, 1.541, and 1.535 eV for MN-EMPA, MN-IMPA, and MN-PMPA, respectively. Due to the small excess energy ($<3 \text{ eV}$), fragmentation would be minimal and a molecular ion would be expected to be observed for these molecules [20]. The spectral properties of the derivatives were very similar to each other and are mainly determined by the naphthalene chromophore. The ionization energy was also calculated for other NM-NAMs; the values for MPA, the final product of sarin, soman, and VX, and for ethyl dimethylaminophosphoric acid (EDMPA) and ethyl phosphorocyanidate (EPC), intermediate products from tabun, and for phosphoric acid (PA), the final product of tabun, were calculated using B3LYP/cc-PVDZ to be 7.871, 7.612, 8.015, and 8.061 eV, respectively (7.958, 7.716, 8.096, and 8.143 eV, respectively, using B3LYP/cc-PVTZ). The rationale for characterizing the other NAMs remained unchanged, although a smaller molecule would have a slightly higher *IE*. These results suggest that all of the MN-NAMs are efficiently ionized through a RE2PI process.

3.2. Extraction

Because a NAM molecule contains polar functional groups (e.g., phosphonic acid) and is soluble in a polar solvent. Then, non-polar n-hexane was used as an extraction solvent to remove non-polar interferences in the sample solution. Subsequently, a slightly polar solvent of diethyl ether was used for extraction of polar NAMs and for better phase separation against the aqueous solution. It should be noted that diethyl ether evaporates easily at lower temperatures (boiling point, 34.6 °C). This permits the sample to be easily concentrated and then dissolved in a solvent suitable for a

derivatization reaction. The recoveries measured for EMPA, IMPA, and PMPA in the urine sample were in the range of 70~73%, which were comparable or slightly better than the value of 46~107% reported in a reference [10].

3.3. Derivatization

Due to a low reactivity in diethyl ether, the analytes were dissolved in acetonitrile. The parameters affecting the derivatization reaction were investigated and the results are shown in Fig. 4. The signal intensity increased rapidly from 55 °C and approached a constant value at temperatures above 65 °C, suggesting that the derivatization reaction reached completion after 5 h at 65 °C. Since the boiling point of acetonitrile is 83 °C, the derivatizations were carried out at 70 °C in this study. The effect of reaction time was investigated at this temperature, and the results indicate that the maximum value was reached after 5 h as shown in Fig. 4(B). No attempt was made to decrease the reaction time: it would be possible to decrease the time to <1 h by slightly increasing the reaction temperature. The effect of the BMN concentration was also investigated at a reaction temperature of 70 °C after a reaction time of 5 h. As shown in Fig. 4(C), the signal intensity was saturated at a concentration ratio above ca. 4-fold for BMN, suggesting completion of the chemical reaction. Accordingly, the reaction temperature, the reaction time, and the concentration of BMN were adjusted at 70 °C, 5 h, and 4 µg/mL, respectively, in this study.

3.4. Two-dimensional GC-MS display

A two-dimensional GC-MS display for a sample mixture containing EMPA, IMPA, and PMPA in human urine is shown in Fig. 5. Molecular ions as well as fragment ions were clearly observed for MN-EMPA, MN-IMPA, and MN-PMPA (see the square parts specified by a white solid line), and are

more clearly shown in the expanded views, thus providing a reliable tool for analysis of NAMs. When PFBBBr was used as the derivatizing reagent for the NAMs (IMPA and PMPA), large signals arising from numerous interferences (mainly from side-reaction products) appeared in the two-dimensional display [20]. On the other hand, as shown in the expanded views in Fig. 5, isotopomers, i.e., $[M]^+$ and $[M+1]^+$ containing zero or one atom of ^{13}C in a molecule, are observed. In fact, MN-EMPA and MN-IMPA give rise to two signal peaks at $m/z = 264, 265$ and $m/z = 278, 279$, respectively. Thus, the signals with retention times of 21.8 and 22.0 min are assigned to MN-EMPA ($m/z = 264$) and MN-IMPA ($m/z = 278$), respectively. The retention time of MN-EMPA is very close to that of MN-IMPA even using GC because of a slight difference in the structure of the side chain (ethyl- or isopropyl-). Note that the signal for MN-PMPA is split into two peaks, with retention times of 23.8 and 24.0 min, which correspond to diastereomers of MN-PMPA separated by GC; they consist of isotopomers, i.e., $[M]^+$ and $[M+1]^+$ at $m/z = 320$ and 321 , respectively. The signals observed at a retention time of 17.5 min (see the yellow circle specified by a solid line) at $m/z = 220$ and 222 correspond to BMN containing ^{79}Br and ^{81}Br in the molecule, respectively. In addition, several peaks were observed, e.g., at $m/z = 185$ and 186 , which can be attributed to products produced by the reaction of BMN and acetonitrile, $[(\text{C}_{10}\text{H}_7)\text{-NH-COCH}_3]^+$ and $[(\text{C}_{10}\text{H}_7)\text{-O-COCH}_3]^+$. They are, however, completely separated by GC and do not interfere with the determination of the NAMs. The total ion chromatogram (TOC) shown at the top of the figure suggests that the NAMs are sufficiently separated by GC, although the background signal cannot be completely negligible in the TOC.

3.5. Mass spectrum

The mass spectra of MN-EMPA, MN-IMPA, and MN-PMPA are shown in Fig. 6. These compounds decomposed into several fragment ions, i.e., $[(\text{C}_{11}\text{H}_{11})\text{-CH}_3\text{PO}_3\text{-}]^+$ ($m/z = 235$), $[(\text{C}_{11}\text{H}_{11})\text{-}$

O-]⁺ ($m/z = 157$), and [(C₁₁H₁₁)-]⁺ ($m/z = 141$), due the fact that their chemical structures are similar. As a result, it would be difficult to identify these compounds by MS when no molecular ion was observed. Fragmentation is accelerated by following two factors: (1) the excess energy in the ionization process (2) the presence of a bulky side chain in a molecule [20]. As discussed above, the excess energy for these compounds is small and the relatively enhanced fragmentation for MN-PMPA would be attributed to the presence of a bulky side chain $-(\text{CH}_3)\text{-HC-C}(\text{CH}_3)_3$ for MN-PMPA.

3.6. Chromatogram

Figure 7 shows the chromatogram obtained by the selected ion monitoring (SIM) of a molecular ion of MN-EMPA, MN-IMPA, and MN-PMPA for a sample mixture containing EMPA, IMPA, and PMPA. As described, MN-PMPA consists of two diastereomers, resulting in two signal peaks in the chromatogram. It should be noted that small signals appeared, especially when monitored at $m/z = 264$ and 278 , for determination of MN-EMPA and MN-IMPA, respectively. These signals appear to arise from the fragments of large molecules such as MN-PMPA, side-reaction products, and impurities in the urine sample. Therefore, excellent performance in separation by GC and detection of the molecular ion by MPI/TOF-MS are both desirable factors to use this approach for the trace analysis of NAMs.

3.7. Selectivity and sensitivity

Figure 8 (A) shows the expanded view of the two-dimensional GC-MS display obtained for PMPA prepared at 20 ng/mL in a human urine sample. The signals of molecular ions were clearly separated against impurities/side-reaction products as shown in Fig. 8 (B), suggesting excellent selectivity of the present method. This favorable result was obtained by the two-step extraction

process using n-hexane and diethyl ether and by superior performance of femtosecond REMPI to observe the molecular ions. From the baseline noise of the background signal observed in Fig. 8 (C), the limit of detection (LOD) was calculated to 0.23 ng/mL for PMPA. Similar experiments were performed for IMPA and EMPA, the LODs being 0.23 and 0.14 ng/mL, respectively. These results were similar to the values of 0.25, 0.24, and 0.22 ng/mL obtained for PMPA, IMPA, and EMPA, respectively, from the chromatograms (see Fig. 8 (D) for PMPA). A straight analytical curve was constructed in the range of 0~70 ng/mL, the R^2 value being 0.95. The LODs obtained by GC-EI/MS were reported to be 50 ng/mL and 2.5~10 ng/mL in full-scan and SIM modes, respectively, and the values were reduced to 60 pg/mL by GC-NICI/MS (the sample was concentrated 1.3~3.3-fold and the SIM mode was used in the measurement) [26]. The LODs measured by GC-PICI/MS-EIMS was reported to be 4 ng/mL [11]. Thus, the LODs achieved in this study (full scan mode) were lower than those obtained using conventional GC-EI/MS and GC-PICI/MS-EI/MS. It should be noted that no appreciable interference was observed in the data shown in Fig. 8. Thus, further concentration of the sample solution (e.g., from 1 mL to 100 μ L by reducing the sample volume) would provide comparable or lower LODs (full-scan mode) than the values obtained by GC-NICI/MS (SIM mode). Thus, the present approach would be useful for reliable trace analysis of NAMs in forensic science.

3.8. Advantage

In this study, diethyl ether was employed as the extraction solvent and a sufficient recovery was obtained for NAMs in the human urine sample. The efficiency of derivatization by BMN is saturated at the present conditions (see Fig. 4), and the signal can be significantly enhanced by means of RE2PI at 267 nm. As a result, in addition to fragment ions, molecular ions for these derivatives were observed as major signals. Moreover, the background signals arising from interferences in urine and side-

reaction products that appeared in the derivatization process were suppressed and also completely separated by GC. Note that it would be possible to separate the enantiomers for each diastereomer (four isomers in total) by using a chiral GC column [26]. It is interesting to note that the relative abundance of the enantiomers would depend on the process used to prepare the NAs, thus providing information for identifying the manufacturer of the CWAs. The determination of impurities/side-reaction products in the sample, e.g., MN-EMPA and/or MN-IMPA occurred in the production process of MN-PMPA, would be particularly useful for identifying the NA supplier. These issues can be attributed to the excellent separation provided by GC and by the comprehensive analysis provided by MPI/TOF-MS, which is in contrast to the limited performance in separation by LC and the dominant fragmentation in EI/MS and no or less fragmentation in CI/MS. It is noteworthy that an MS-MS technique was successfully used for the analysis of NAMs [11]. However, this technique would be difficult to use for a comprehensive analysis, preventing the analysis of the constituents including side-reaction products and impurities for identification of criminals.

4. Conclusions

BMN, a new derivatizing agent was used in this study for the determination of NAMs such as EMPA, IMPA, and PMPA based on GC-TOF-MS using a UV femtosecond laser (267 nm) as the ionization source. The derivatized MN-NAMs were separated by GC and the molecular ions were clearly identified by MPI/TOF-MS in addition to fragment ions, allowing the simultaneous, reliable determination and finger-print identification of NAMs. It is well known that a compound with a bromomethyl group ($-\text{CH}_2\text{Br}$) reacts with an exchangeable hydrogen. Then, BNM can be used to derivatize a variety of molecules with polar groups such as $-\text{OH}$, $-\text{COOH}$, and $-\text{NH}_2$ and the naphthalene chromophore in a molecule absorbs UV photons efficiently for RE2PI. Therefore, it

would be useful for the sensitive determination of polar organic compounds such as persistent organic pollutants (POPs) dissolved in environmental water or amino acids and peptides in biological fluids. Thus, the present technique has the potential for use in trace analysis only in forensic science but also in the environmental and biological sciences.

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Figure Captions

Fig. 1. Schematic diagram of the experimental apparatus used in this study.

Fig. 2. Sample preparation procedure: (A) NAMs were spiked into urine (B) extraction of interferences with n-hexane (C) extraction of NAMs with diethyl ether (D) water in the extract was removed by anhydrous Na₂SO₄ (E) diethyl ether was evaporated and the analytes were dissolved in acetonitrile for derivatization with BMN in the presence of DMF to partly dissolve K₂CO₃.

Fig. 3. Calculated absorption spectra for (A) MN-EMPA (B) MN-IMPA (C) MN-PMPA. *EE*, the lowest transition energy; *IE*, the ionization energy; *IE/2*, half value of the ionization energy.

Fig. 4. Optimization of the derivatization reaction. (A) effect of reaction temperature; reaction time, 5 h; molar concentration ratio of BMN and NAM, 1:1 (B) effect of reaction time; temperature, 70 °C; molar concentration ratio of BMN and NAM, 1:1 (C) effect of molar concentration ratio of BMN and NAM; temperature, 70 °C; reaction time, 5 h. Concentration of NAM in the sample, 1 µg/mL (5.3 nmol/mL).

Fig. 5. Two-dimensional GC-MS display of a sample mixture containing EMPA, IMPA, and PMPA in human urine. A concentration of 500 ng/mL for each compound. Inserts show expanded views of the regions where MN-EMPA, MN-IMPA, MN-PMPA appear. The solid yellow circle shows the region where BMN and decomposed products of BMN and acetonitrile appear. The broken yellow circle shows the fragment ions of MN-NAMs. The total ion chromatogram is shown at the top of the two-dimensional display.

Fig. 6. Mass spectra of a sample mixture containing EMPA, IMPA, and PMPA in human urine. Concentration, 500 ng/mL for each compound. The mass spectrum of MN-EMPA ($m/z =$

264), MN-IMPA ($m/z = 278$), and MN-PMPA ($m/z = 320$) were constructed by extracting the data at retention times of 21.8, 22.0, and 24.0 min, respectively. The signals specified by asterisks appeared from BMN that elutes even after 20 min, probably due to adsorption of BMN on the stationary phase of the capillary column (see the streaks in fig. 5).

Fig. 7. Chromatograms of a sample mixture containing EMPA, IMPA, and PMPA in human urine. Concentration, 500 ng/mL for each compound. The chromatogram of MN-EMPA, MN-IMPA, and MN-PMPA was constructed by extracting the data at $m/z = 264$, 278, and 320, respectively.

Fig. 8. Two-dimensional GC-MS display measured for a urine sample containing (A) PMPA prepared at a concentration of 20 ng/mL (B) no PMPA (blank). (C) mass spectrum (D) gas chromatogram measured for a sample containing (a) PMPA prepared at a concentration of 20 ng/mL (b) no PMPA (blank).

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