

Detection of Bis(diphenylarsine)oxide, Diphenylarsinic Acid and Phenylarsonic Acid, Compounds Probably Derived from Chemical Warfare Agents, in Drinking Well Water

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The inhabitants living in a specific region of Kizaki area in Kamisu-town, Ibaraki Prefecture exhibited uncommon clinical central nervous system symptoms. A graphite furnace atomic absorption spectrophotometer detected markedly elevated concentration of arsenic (4.5 ppm) in their drinking well water. Further investigation using HPLC, GC/MS and HPLC/ICP/MS demonstrated that the structures of the arsenic were bis(diphenylarsine)oxide (BDPAO), diphenylarsinic acid (DPAA) and phenylarsonic acid (PAA), compounds that can be derived from the chemical warfare agents, diphenylchloroarsine (DA) and diphenylcyanoarsine (DC). The predominant form of the arsenic compound in the well water was DPAA (maximum 15 ppm), so that it was calculated that the inhabitants ingested 11–30 mg of DPAA daily. This is the first report of inhabitants that were injured by drinking well water contaminated with organic arsenic compounds that were likely derived from chemical weapons.

Key words — bis(diphenylarsine)oxide, diphenylarsinic acid, phenylarsonic acid, fragment of chemical weapon, phenylated arsine, well water

INTRODUCTION

In 2002, the inhabitants lived in the southwestern part of Kizaki area of Kamisu-town, Ibaraki Prefecture, began developing uncommon clinical symptoms of the central nervous system, including walking difficulty, tremor of the extremities, dysarthria, myoclonus, double vision, insomnia and memory disturbance.¹⁾ A characteristic common observation in the patients was that the symptoms improved or disappeared after a few weeks of separation from the residential area, and recurred within a few months of their return to their houses. In addition to humans,

dogs and hamsters kept in this area died one after another during the past few years. Since all patients and these animals ingested well water from the same origin, we suspected that the water was contaminated with a toxic substance(s).

On March 19, 2003, we first analyzed the well water by use of a graphite furnace atomic absorption spectrophotometer and detected arsenic levels of 4.5 ppm that was 450-times higher than upper limit of the water quality standard. Further investigation demonstrated that the arsenic consisted of bis(diphenylarsine)oxide (BDPAO), diphenylarsinic acid (DPAA) and phenylarsonic acid (PAA) that can be derived from chemical warfare agents, diphenylchloroarsine (DA) and diphenylcyanoarsine (DC).^{2–4)}

In this report, we describe the methods that proved the toxic pollutants to be the organic arsenic compounds.

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Table 1. Analytical Conditions for Determination of Total Arsenic by Graphite Furnace Atomic Absorption Spectrophotometer

	furnace temperature (°C)	Operating time (s/min)	Argon gas flow rate (l/min)
Drying	100	20	2.0
Ashing	900	15	2.0
Atomizing	2400	10	0.6
Cleaning	2700	2	2.0

Instrument: Seiko instruments: SAS7500 equipped with graphite furnace atomizer PS200.

MATERIALS AND METHODS

Well Water — Well water for inspection was collected in arsenic free glass bottles or polyethylene containers from the faucets in a multiple dwelling house, and was preserved at 4°C.

Chemicals — BDPAO (98% purity) and DPAA (99%) were synthesized by Hodogaya Contract Laboratory (Ibaraki, Japan). Magnesium nitrate hexahydrate was purchased from Merck (Germany) and the solid phase column (POLYSorb MP-DVB) was from Transgenomic Inc. (Omaha, NE, U.S.A.). All other reagents and solvents were of analytical grade. Mg²⁺ solution (1.25%, w/v) was prepared by dissolving 132 g of Mg(NO₃)₂·6H₂O into 100 ml of water.

Apparatus and Operating Conditions — To detect BDPAO by GC/MS, an M-9000 instrument equipped with a library of NIST 129000 (Hitachi High-Technologies corp., Tokyo, Japan) was used. A DB-5ms (30 m × 0.25 mm i.d., 0.25 μm film thickness) column was used (J & W Scientific, Folman, CA, U.S.A.). The operating conditions were as follows: the injection port temperature, 250°C; the flow-rate of the carrier gas (helium), 1.5 ml/min; and the column oven temperature was programmed from 50 to 160°C at 10°C/min after a 1-min delay from the start time, from 160 to 210°C at 8°C/min, from 210 to 250°C at 4°C/min, from 250 to 300°C at 6°C/min after a 3-min keeping at 250°C, and at 300°C for 15 min. DPAA (as diphenyl(propylthio)arsine and PAA {as bis(propylthio)phenylarsine} and were assessed by GC/MS in the electron ionization (EI) mode using a QP-5000 instrument (Shimadzu, Kyoto, Japan) with a DB-5ms column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The operating conditions of the EI-MS (SHIMADZU GC/MS QP-5000) were as follows: the flow-rate of the carrier gas (helium), 0.8 ml/min; column oven temperature, was programmed from 60 to 280°C at 10°C/min after a 1-min delay from the start time, and main-

tained at 280°C for 10 min; injection port temperature, 250°C; and ionization energy, 70 eV. The multiple ion detector was focused at *m/z* 227, 261 and 304 for DPAA and at *m/z* 227, 259 and 302 for PAA.

HPLC was performed using an LC-10A series (Shimadzu) equipped with diodearray detector SPD-M10A. The column used was an L-Column ODS (4.6 × 150 mm) (Chemicals Evaluation and Research Institute, Tokyo, Japan).

The atomic absorption spectrophotometer used was an SAS 7500 equipped with graphite furnace atomizer PS200 (Seiko instruments Inc., Chiba, Japan). Inductively coupled plasma-mass spectrometry (ICP/MS) and HPLC/ICP/MS were performed by an ICP-3DQMS P-5000 equipped with an HPLC L-7000 series (Hitachi High-Technologies corp.).

Analysis of Japanese Standard Items in Well Water — Standard items except for arsenic, *e.g.* pH, potassium permanganate consumption, heavy metals, trace elements, pesticides and volatile organic halogen compounds in well water were determined according to the Standard Methods for Examination of Water in Japan.⁵⁾

Analysis of Total Arsenic in Well Water — Mg²⁺ solution (1.25%, w/v) was added to the sample to give a final concentration of 0.025% Mg²⁺ in the mixture, and an aliquot was used for the measurement. Total arsenic concentration was determined using a graphite furnace atomic absorption spectrophotometer according to the method described by Ishizaki *et al.*⁶⁾ (Table 1).

Preliminary Characterization of Inorganic and Organic Arsenic — Inorganic arsenic levels in well water were measured by the method of Ishizaki *et al.*^{7,8)} Ten milliliters of 10 N HCl and 1 ml of 40% potassium iodide were added to 0.5–1.0 ml of well water to reduce As(V) to As(III). Inorganic arsenic was extracted twice with 5–10 ml of chloroform. Both extracts were combined, and arsenic was extracted with 1 ml of an aqueous solution of magnesium salt (0.025%, w/v), and an aliquot was used

for the measurement. The inorganic arsenic concentration in the extract was determined by using a graphite furnace atomic absorption spectrophotometer. The organic arsenic level in the well water was calculated by subtracting the inorganic arsenic amount from the total arsenic amount.

Analysis of BDPAO by GC-MS — BDPAO was purified from well water by solid phase extraction methods for pesticide described in the Standard Methods for Examination of Water.⁴⁾ Briefly, 500–1000 ml of sample was applied to a solid phase column cartridge at a flow-rate of 20 ml/min. After the column was air dried, BDPAO was eluted with 4 ml of dichloromethane. The eluate was evaporated to dryness under a nitrogen stream. The residue was dissolved in 0.5 ml of acetone, and an aliquot was analyzed by GC/MS in the EI mode.

Determination of DPAA by HPLC — DPAA was separated on an L-Column ODS. The mobile phase consisted of solvent A (0.05% aqueous trifluoroacetic acid) and B (acetonitrile) according to the gradient presented in Table 2. The injection

volume was 20 μ l, the column oven temperature was maintained at 35°C, and the flow-rate of the mobile phase was set at 0.8 ml/min.

Determination of PAA by GC-MS — PAA in well water was measured by GC/MS with selected-ion monitoring (SIM) after conversion into propanethiol derivatives, as described by Hanaoka *et al.*⁹⁾ PAA was changed into bis(propylthio)phenylarsine by the following derivatization procedure. One milliliter of 2 N HCl and 50 μ l of n-propanethiol were added to 10 ml of well water, and the mixture was shaken intensely in a plug-sealed test tube. After the test tube was left for 10 min, followed by centrifugation at 1500 \times g for 5 min, the reacted organic compounds were extracted twice with 10 ml of n-hexane. The extracted n-hexane layers were combined, concentrated to 10 ml under nitrogen stream, and an aliquot was analyzed by GC-MS.

Detection of DPAA and PAA by HPLC/ICP/MS — The operating conditions for the ICP-MS instrument are presented in Table 3. These conditions were obtained after preliminary optimization of the instrument parameters. The typical chromatogram of these compounds was shown in Fig. 4.

Table 2. Mobile Phase Gradient for Determination of DPAA by HPLC

Time/min	Solvent A, %	Solvent B, %
0	95	5
10	95	5
20	60	40
28	60	40
29	5	95
35	5	95
36	95	5
50	95	5

RESULTS AND DISCUSSION

The well in which the arsenic compounds were detected was drilled in reclaimed land. This area is a reburied region after gravel picking. The depth of the well was about 15.5 m, and the water was pumped up from points that are about 6 and 12 m in depth. In this study, we used water obtained from

Table 3. Operating Conditions of HPLC-ICP-MS Instruments for Detection of DPAA and PAA

ICP-MS conditions	
RF power	1.2 kW
Plasma gas flow rate	14 l/min
Auxiliary gas flow rate	1.2 l/min
Nebulizer gas flow rate	1.3 l/min
Dwell time	500 ms
Monitored mass	As (<i>m/z</i> 75)
HPLC conditions	
Analysis for diphenylarsinic acid	
Column	Shodex RSpak NH-814 (8.0 \times 250 mm)
Column temperature	40°C
Flow rate	1.0 ml/min
Mobile phase	5 mM nitric acid, 8 mM ammonium nitrate

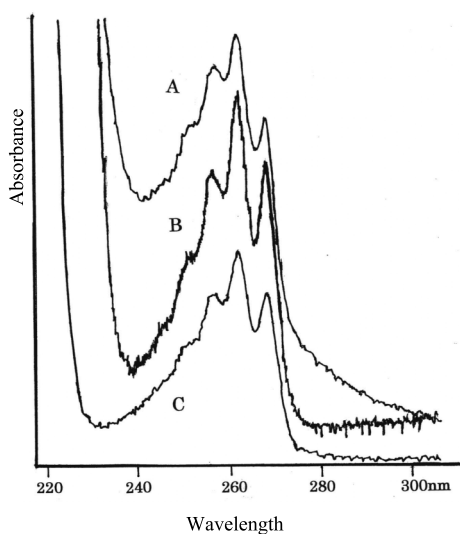


Fig. 1. UV Spectra of Well Water and Authentic Phenylated Arsenic Compounds

A, well water; B, DPAA; C, PAA.

the 12 m depth. The pH of the well water was 7.3, and all inspection items (potassium permanganate consumption, heavy metals, volatile organic halogen compounds, organochloric pesticides and organophosphoric pesticides) were less than the reference value of water quality standards for drinking water, with the exception that an abnormally high concentration (4.5 ppm) of total arsenic was detected.

The Preliminary Test for Detecting Organic Arsenic

Our spectrophotometric analysis demonstrated that the inorganic arsenic concentration was 1.5 ppm. Since the total arsenic concentration was 4.5 ppm, the organic arsenic level was calculated as 3.0 ppm. Thus, although organic arsenic was suspected as the predominant form of arsenic in this well water, we could not exclude the possibility that the water was also polluted with inorganic arsenic. However, LC/ICP/MS analysis later proved that some phenyl arsenic compounds extracted with organic layer was re-distributed to the aqueous phase during the subsequent extraction procedure. Therefore, it was concluded that all arsenic detected in the well water was derived from organic arsenic compounds.

Ultraviolet Absorption Spectra of the Well Water

To obtain information about the structures of the organic arsenic compounds, the ultraviolet (UV) spectrum of the well water was measured. The well

water showed a UV absorption maxima (λ_{\max}) at 255, 262 and 268 nm characteristic of phenyl derivative compounds¹⁰⁾ (Fig. 1). These results suggested that the organic arsenic compounds in the well water might be phenyl derivatives.

Detection of BDPAO in the Well Water

An unknown prominent peak which demonstrated a molecular ion at m/z 474 at a retention time of 35.7 min was observed by GC-MS analysis according to the Standard Methods for Examination of Water.⁵⁾ The mass spectrum was compared with the library (NIST 129000) included in the mass spectrometric analysis system, and was identified as BDPAO with a high probability (71% rate of concordance). Furthermore, the mass spectrum and retention time of the BDPAO in the well water was completely superimposable over that obtained for authentic BDPAO (Fig. 2). Thus, the structure of this compound in the well water was unequivocally confirmed as BDPAO. Haas *et al.* demonstrated that BDPAO was a breakdown product of the chemical weapons, DA and DC.²⁾ DA and DC are hydrolyzed to diphenyl arsine hydroxide, which is then converted to BDPAO (Fig. 3). In addition, BDPAO has been reported to be further hydrolyzed into DPAA.²⁾ In contrast to DPAA, the origin of PAA has not been clarified, but there are two possibilities that¹⁾ PAA may have been produced by hydrolysis of phenyldichloroarsine (PDCA) which is a putative by-product in synthesizing DA or DC,¹¹⁾ and²⁾ as suggested by Kohler *et al.*,⁴⁾ degradation of DPAA by bacteria in soil might have produced PAA. Although the present method was not suitable for quantification of BDPAO, the concentration was calculated to be 0.54 ppm or less (0.17 ppm or less as arsenic) by subtracting DPAA and PAA concentration from the total arsenic level in the same well water.

Detection of DPAA and PAA in the Well Water

If organic arsenic in the well water was truly derived from DA and/or DC, significant concentrations of DPAA should be detected in the same water because DPAA is a major degradation product of BDPAO^{2,9)} and is a more hydrophilic structure compared with the parent BDPAO. As we expected, a high concentration of DPAA (15.0 ppm, 4.3 ppm as arsenic) was determined in the same well water by HPLC analysis. By contrast, PAA was not detected by HPLC, but was able to be analyzed by GC-MS after converting into propanethiol derivatives (Fig. 5). The results demonstrated that PAA concen-

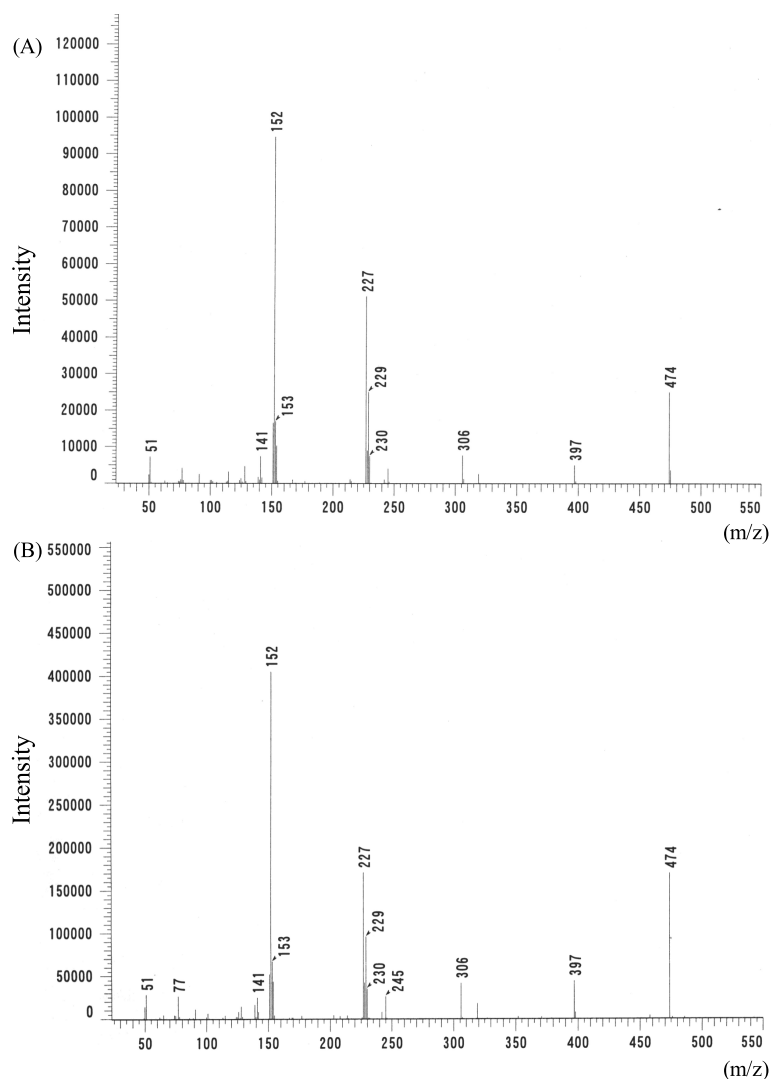


Fig. 2. Mass Spectra of Bis(diphenylarsine)oxide
A, authentic BDPAO; B, BDPAO in well water.

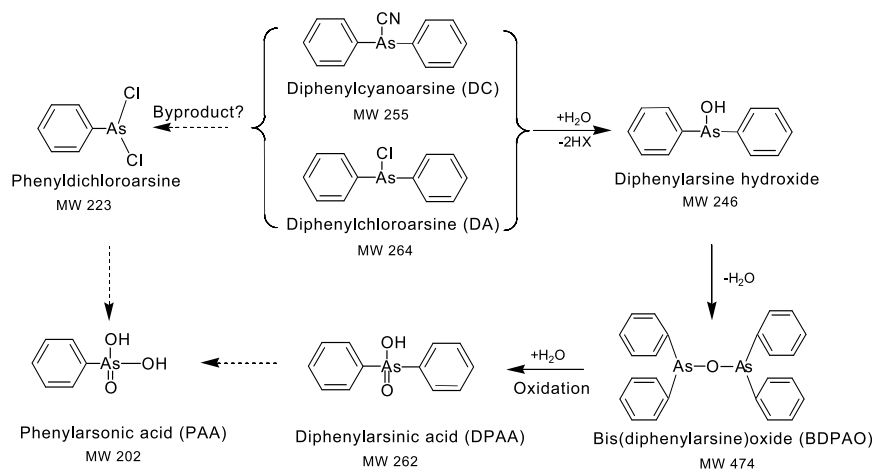


Fig. 3. The Degradation Pathway of DA and DC

Solid arrows represent known degradation steps, while broken arrows represent putative reactions that have yet to be confirmed.

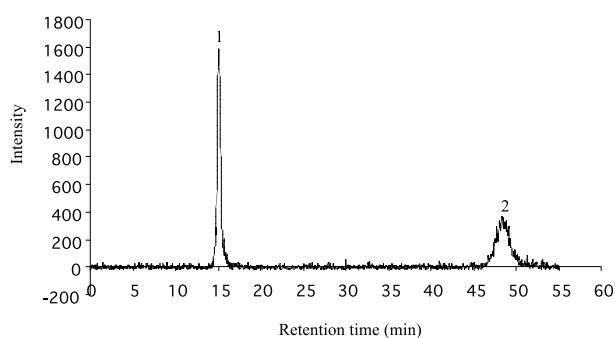


Fig. 4. HPLC-ICP-MS of Diphenylarsinic Acid and Phenylarsonic Acid
1, PAA; 2, DPAA.

tration in the well water was 0.072 ppm (0.027 ppm as arsenic).

Estimation of Daily Arsenic Intake

It could be calculated that the inhabitants ingested 3.4–9.0 mg/day of arsenic because they used to drink 0.75–2.0 liters of the well water daily.¹²⁾ Disturbances in the central nervous system was one of the characteristic features of subacute inorganic arsenic poisoning caused by arsenic contaminated soy-sauce¹³⁾ and dry-milk (Morinaga).^{14,15)} In these cases, people ingested more than 3 mg of inorganic arsenic daily, and Ishinishi *et al.*¹⁶⁾ speculated that subacute

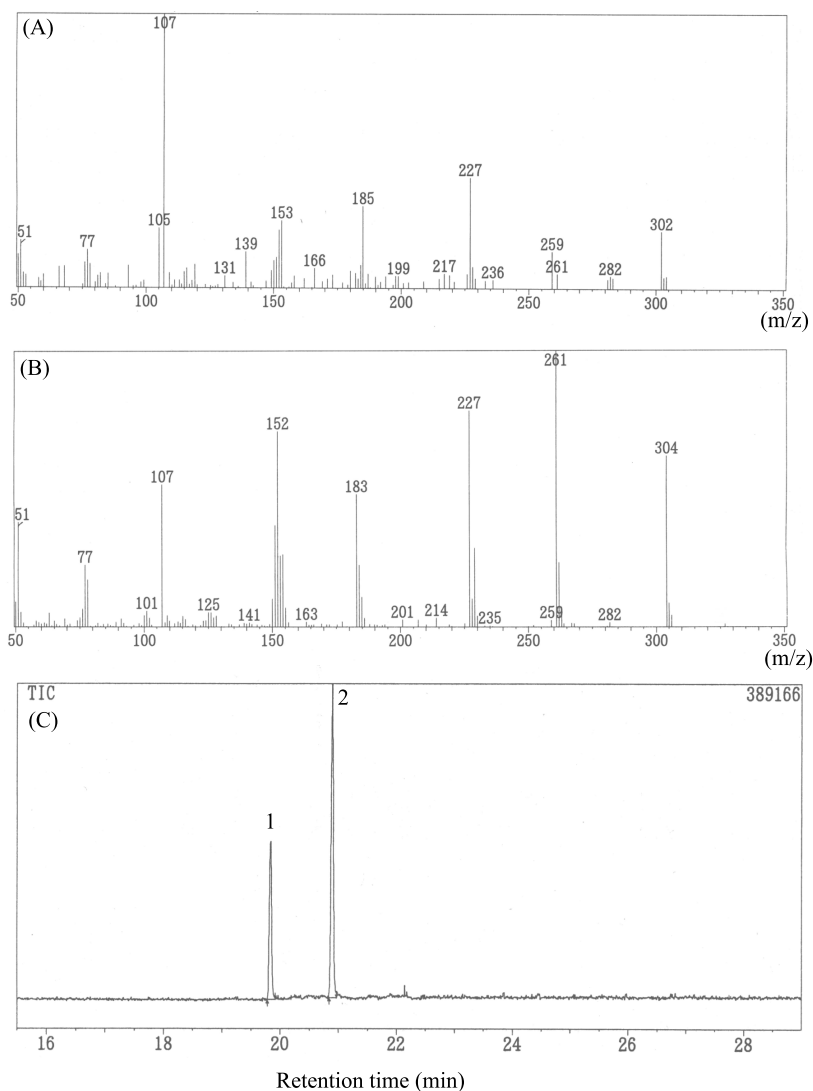


Fig. 5. GC-MS Spectrum of Authentic Bis(propylthio)phenylarsine (A) and Diphenyl(propylthio)arsine (B), and SIM Chromatogram (C) of Bis(propylthio)phenylarsine (1) and Diphenyl(propylthio)arsine (2)

The multiple ion detector was focused at m/z 227, 259 and 302 for bis(propylthio)phenylarsine, and at m/z 227, 261 and 304 for phenylarsonic acid (PAA).

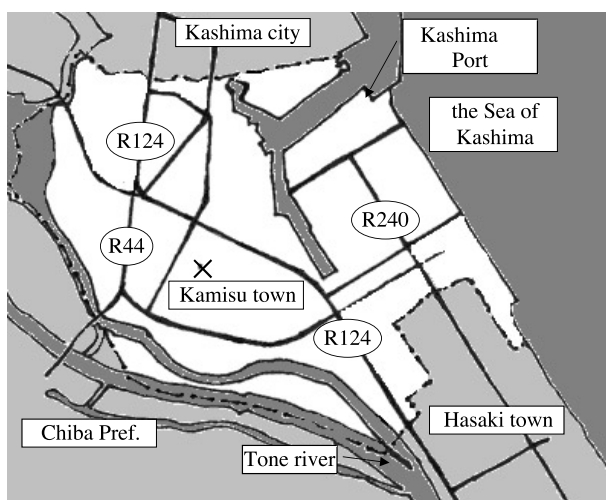


Fig. 6. Detected Location of Phenylated Arsenic Compounds in Kamisu Town

X = the site where DPAA was detected for first time.

inorganic arsenic poisoning occurred when more than 3 mg/day of inorganic arsenic was taken for several weeks. Although it remains controversial whether the symptoms observed in our cases were caused directly by organic arsenic or not, at least the well water did not contain significant amount of inorganic arsenic. Therefore, it is possible that both organic and inorganic poisoning can exhibit similar symptoms in their subacute phase.

In summary, we detected extraordinary concentrations of organic arsenic in well water in Kamisu town, Ibaraki Prefecture (Fig. 6). Further investigation identified the chemical structures of the arsenic as BDPAO, DPAA and PAA that would appear to be breakdown products of chemical warfare agents. This is the first report of poisoning of inhabitants by organic arsenic compounds in drinking well water.

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