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Development of a Method for Analysis of Radionuclides in Biological Samples Using ICP Mass Spectrometer

Akari Homma[1], Hirofumi Tazoe[2], Masatoshi Yamada[2], Shingo Terashima[3] and Yoichiro Hosokawa[3]*

[1]*Department of Radiology, Steel Memorial Muroran Hospital, 1-45 Chiribetsu-cho, Muroran, Hokkaido, Japan*
[2]*Department of Radiation Chemistry, Institute of Radiation Emergency Medicine, Hirosaki University, 66-1 Hon-cho, Hirosaki, Aomori, Japan*
[3]*Department of Radiation Science, Hirosaki University School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori, Japan*

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Internal ionizing radiation exposure dose is estimated indirectly by bioassay of biological samples such as feces and urine. In order to establish a method for internal exposure dose estimation for urine samples, we evaluated sample pretreatment by wet digestion prior to radionuclide measurement by inductively coupled plasma mass spectrometry. Wet digestion method was selected so that not only thorium and uranium but also ^{90}Sr and ^{99}Tc in urine samples could be determined. Analysis of thorium and uranium in tap water of Hirosaki city, and of mineral water and urine samples provided by the residents of Fukushima prefecture were performed using the resulting bioassay protocol. The concentrations of ^{232}Th in urine samples were 4.8 to 43.1 ppt and ^{238}U concentrations were 25.8 to 133.2 ppt, which were higher than those in the corresponding water and mineral water samples. These values are thought to be affected by the thorium and uranium content of both food and drinking water. Pretreatment of urine samples for thorium and uranium for inductively coupled plasma mass spectrometry was established as a bioassay for internal exposure dose estimation.

Key words: ICP-MS, Wet digestion system, Uranium, Urine sample

1. Introduction

Internal exposure to radionuclides resulting from natural sources or man-made nuclear activities such as atomic bomb tests and nuclear terrorism may occur when people absorb radioactive materials via routes such as the percutaneous, oral, and respiratory. In order to evaluate this dose, it is necessary to estimate the radioactivity in the body, and one approach is the bioassay method. Biological samples such as feces and urine are chemically treated before radioactivity measurement, allowing the detection of trace amounts of radioactive isotopes and indirect estimation of internal exposure dose to alpha or beta particles with a short travel range in the body. Urine is simple to collect and is used for research as a general biological sample. Depending on the passage of time after ingestion of radioactive materials, by calculating the percentage of radioactive material excreted from the body, it is possible to calculate intake from the excreted amount of radioactivity[1)].

The radionuclide ^{238}U, which decays to ^{234}Th, has a

*Yoichiro Hosokawa: Department of Radiation Science, Technology, Hirosaki University School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori, Japan
E-mail: hosokawa@ hirosaki-u.ac.jp

long half-life of 4.468×10^9 years. In water, uranium is present as various anionic forms and thorium is present as colloidal $Th(OH)_4$. It was reported that health effects on kidney function following ingestion of uranium are due to chemical toxicity. Uranium concentrations in drinking water and daily intake of uranium were statistically significantly associated with calcium fractional excretion[2]. In addition, thorium and uranium concentrations in drinking water differ depending on water hardness and source with large regional differences reported even within Japan[3]. Furthermore, thorium and uranium contained in foods routinely ingested by Japanese people vary with food type; 60% of ^{232}Th obtained from food is ingested from eggs and dairy products while 70% of ^{238}U is ingested from seaweeds such as kelp[4]. Regular, daily intake of these food sources is reflected in thorium and uranium urine concentrations. Uranium and its fission products are important elements from the viewpoint of nuclear fuel that have harmful effects on the human body due to alpha particle emissions. The potential for contamination following the accident at the TEPCO Fukushima Daiichi Nuclear Power Plant (FDNPP) on March 2011, which used uranium enriched with 3.4 to 3.7 wt% ^{235}U[5], was therefore of concern. However, uranium contamination was not observed in the soils and rivers following the incident[6].

Hirosaki University has continued to carry out screening and environmental surveys for the effects of radiation using biological samples. Shi *et al*. reported that uranium, thorium, and plutonium isotopes in urine samples could be rapidly and accurately determined by inductively coupled plasma mass spectrometer (ICP-MS) after the pre-treatment, which separates complicated matrices and concentrates the analyte by coprecipiation method[7]. In this method, uranium, thorium, and plutonium isotopes in urine were subjected to analysis, whereas, radionuclides, such as ^{90}Sr and ^{99}Tc, with high water solubility and low concentrations were excluded. In this research, we aimed at determining radionuclides, such as uranium and thorium, including ^{90}Sr and ^{99}Tc, that require concentration and chemical separation; we selected the wet digestion method as a pretreatment method to make it possible. As the first step, we designed this study to establish an internal exposure evaluation method for uranium and thorium in urine samples obtained from the residents of Fukushima prefecture. We evaluated the sample pretreatment method and the characteristics of the equipment used for digestion of samples arriving at a working protocol. Because the radiation dose level of the biological sample is very small, it is necessary to use a radiation detector with high detection sensitivity and low background. ICP-MS was selected as the measurement technique, and thorium and uranium in urine samples were measured after applying the digestion protocol. The nuclide concentrations of ^{232}Th and ^{238}U in water and urine samples were evaluated.

Table 1. Urine sample collection date and gender

Sample ID	Date	Gender
U4	No data	No data
U7	2011/4/13	Male
U8	2011/4/14	Male
U9	2011/4/15	Male
U10	2011/4/15	Male
U11	2011/4/15	Female

2. Materials and Equipment

Samples and Reagents

Tap water and five types of mineral water from the city of Hirosaki were used as comparative drinking water samples. Urine samples were collected from the residents of Fukushima prefecture in April 2011 (Table 1). In addition, 100% orange Juice produced by Morinaga Milk Industry (Kanagawa, Japan) as a liquid containing organic matter was used for pilot analysis.

Ultrapure water (Milli-Q water) was obtained from a Milli-Q Integral 3 system (Merck Millipore, Germany). Nitric acid used for sample digestion was of electronic industry grade (Kanto Chemicals, Tokyo, Japan). XSTC-13 multi-elemental solution was obtained from Spex CertiPrep (NJ, USA) and used as a standard reference solution for ICP-MS. Thorium and uranium concentrations in this standard solution were 10.10 ± 0.05 and 10.00 ± 0.05 mg/mL, respectively.

Instruments

(1) Wet digestion system

It is necessary to measure urine sample after digestion of the organic matter of the sample. Wet digestion is a method of chemical degradation and dissolution of organic matter by adding an oxidizing acid; normally a high concentration of nitric acid, in conjunction with hydrogen peroxide is used with heating. The wet digester (SpeedDigester, K-439, BÜCHI, Flawil, Switzerland) can treat six specimens in special test tubes at the same time. The upper parts of the test tubes are connected by an exhaust manifold. There is an inlet next to test tube 1 and the structure is evacuated from the exhaust port near test tube 6 (Fig. 1). Consequently, test tube 1 is positioned on the upstream side and the opposite test tube 6 is on the downstream side of the exhaust airflow. Considering the heated part of the sample to be that inserted inside the insulation, we determined the maximum solution volume that can be processed to be 100 mL. As NO_x and CO_2 are generated from the digestion of organic matter in addition to water vapor and nitric acid gas from the

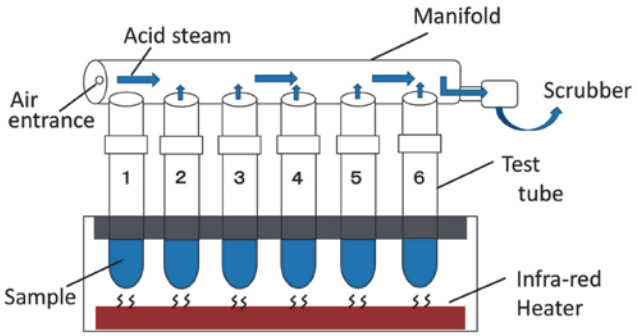

Fig. 1. Overview of the wet digestion system.

Table 2. ICP-MS conditions for analysis

RF power /watt	1550
Sampling depth /cm	8
Carrire gas /L min^{-1}	1.0
Nebulizer	Micro-Mist
Spray chamber	Quartz Scott-type double pass
Mass to charge ratio m/z	203, 205, 232, 234, 235, 238,
Number of sweeps	100
Integration time /sec	3.0 (1.0 sec for m/z = 203, 205)
Number of measurements	3

sample mixed and heated with nitric acid, it is necessary to safely isolate and process these exhaust gases. In order to safely discharge the acid vapor, a cooling device and an alkaline scrubber were connected to the exhaust port. Furthermore, a ribbon heater was wound around the top of the test tubes and manifold to prevent heat radiation.

During the initial stage of digestion, there is a possibility that the sample may be scattered by bubbling due to rapid digestion of organic matter or by boiling caused by excessive heating. Since the temperature control of this digester utilizes a thermocouple installed near the heater, the set temperature of digestion system is different from the solution temperature in the test tube. Therefore, the set temperature was investigated to prevent excessive heating of the sample (Study 1). In addition, (Study 2), the evaporation time at each set temperature was investigated. Since the upper part of each test tube is connected by a manifold there is a possibility of cross contamination and this was also evaluated (Study 3).

(2) ICP-MS

Thorium and uranium were determined using an inductively coupled plasma mass spectrometer, ICP-MS (Agilent 8800, Agilent Technologies), a technique which has been greatly developed in recent years (Table 2). ICP-MS uses a high temperature (6000 K) plasma (ICP), generated by applying high frequency electric field to Ar gas as an ion source and detects generated ions of a specimen. It is possible to achieve a detection limit of about 0.03 ppt for nuclides such as uranium and thorium with long half-lives. Analysis time is about three minutes per sample including measurement and washing.

3. Methods

3.1. Set temperature vs sample temperature in the wet digestion system (Study 1)

Ultrapure water, 100 mL, was placed in a test tube and the temperature of the solution before heating was measured with a mercury thermometer. After placing the test tube in the digestion system, the set temperature was gradually increased from 110 ℃, and the solution temperature at each setting was measured.

3.2. Evaluation of evaporation time (study 2) and cross contamination (study 3) in the wet digestion system

The empty weight of six test tubes was measured and 50 mL of orange juice sample and 50 mL of 61% nitric acid (1: 1 v/v), were added to all six test tubes. One gram of X-STC-13 standard solution containing about 10 ppm of thorium and uranium was added to test tubes 1, 3, and 5. Test tubes 2, 4, and 6 were used as blank samples. X-STC-13 standard solution samples and blank sample tubes were placed alternately around the manifold and heated to the set temperature of 200 ℃ determined by study 1. The digestion was interrupted at regular intervals and after about 30 minutes of cooling on each occasion the weights of each of the test tubes was measured. These cycles were repeated until evaporation to dryness was complete.

After complete evaporation, 5 mL of 61% nitric acid was added to each of the sample tubes and further heated at 200 ℃. Digestion of organic substances was carried out three times in the same manner until the dry matter in all of the test tubes became white. After completion of digestion, 20 mL of 12% nitric acid was added to the dried matter and the tubes heated for 1 hour for dissolution. The degraded sample was transferred from each test tube to a secondary container. Then the tubes were washed using a small amount of ultrapure water so as to prevent sample loss; then, the above digestion operation was repeated on this solution. In order to obtain thorium and uranium concentrations suitable for measurement, X-STC-13 standard solution samples were diluted 10,000 times with 3% nitric acid and blank samples diluted 10 times prior to ICP-MS. Thorium and uranium concentrations were measured by absolute calibration-curve method.

Table 3. Components of simulated urine sample

Component		conc. /g L^{-1}
Inorganic	Organic	
Na^+		5.4
K^+		0.2
Mg^{2+}		0.65
Ca^{2+}		0.2
Cl^-		9.6
SO_4^{2-}		1.35
	Urea	17

3.3. Analysis of simulated urine samples

For validation of the proposed method, simulated urine samples were prepared using salts and urea as an organic component similar to the typical human urine. Known concentrations of U and Th were artificially spiked in simulated urine.

Matrix matching simulated urine sample consisted of a saline solution (including Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}) at a concentration of 17.4 g of salts L^{-1} and urea at a concentration of about 17 g L^{-1}, based on the NUSIMEP-4 interlaboratory comparison campaign. Urea concentration is lower than usual, but to simplify the organic composition, only urea was used. The urea used for the urine preparation was from Wako Chemicals. Firstly, salt and urea were dissolved in 3 M HNO_3 and purified by passing through U-TEVA resin columns (Eichrom Technologies, USA). Purified sample was diluted using Milli-Q water to adjust the concentration (Table 3). Finally, Thorium and uranium were spiked to simulated urine samples.

3.4. Analysis of water and urine samples

Water samples, 80 mL in each case, were filtered with membrane filters (ϕ47 mm in diameter; 0.45 μm-pore), then acidified by adding 3 mL of 61% nitric acid. These were measured directly by ICP-MS. Thorium and uranium concentrations were measured by absolute calibration-curve method.

A maximum volume of 100 mL of urine samples may be used. Therefore, 50 mL samples were taken and wet digestion was performed as described above for orange juice samples (50 mL of urine sample and 50 mL of 61 % nitric acid; 1: 1 v/v). Wet digestion was performed twice at 200℃, including washing of the test tube with ultrapure water. After evaporation, digestion of organic matter was carried out once with 5 mL of 61% nitric acid and it was confirmed that the color of the sample was white. Following complete digestion, 20 mL of 12% nitric acid was added to the dried matter and heated for 1 h for dissolution. The sample was filtered and the final volume was adjusted to 100 mL with ultrapure water for analysis. Treated urine samples can be injected into the ICP-MS in Ultra-Robust mode. This analysis mode can improve the plasma robustness, which reduces matrix suppression. Furthermore, a known amount of thallium standard (Tl) (Wako Chemicals, Japan) was added to each sample and standard for the internal standard method, and the U and Th concentrations in the sample were calculated by comparing the Tl in the sample with the standard. Tl concentration was 50 ppb in the final solution. The instrument was tuned on a daily basis to ensure optimization. Dwell times were 10 ms for ^{205}Tl, and 30 ms for ^{232}Th and ^{238}U. A hundred sweeps were carried out per replicate (3 replicates per sample). Figure 2 shows a flow chart of this analytical procedure.

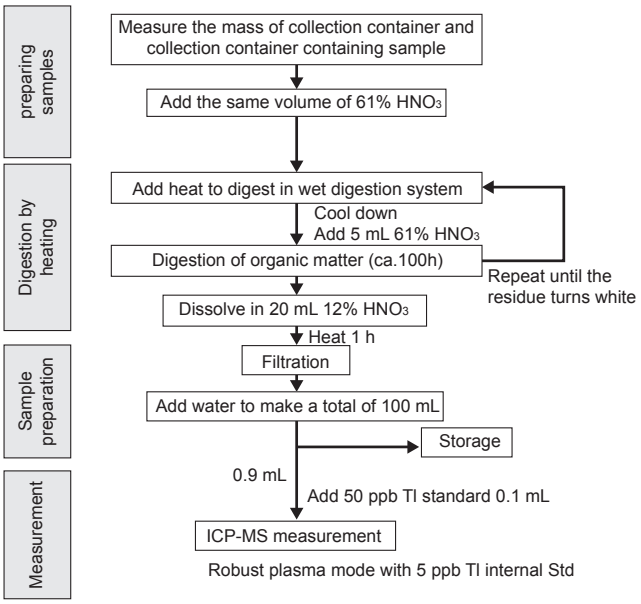

Fig. 2. Flow chart of the analytical procedure for the determination of ^{232}Th and ^{238}U in urine samples by ICP-MS.

4. Results and Discussion

4.1. Evaluation of set temperature in the wet digestion system (Study 1)

Figure 3 shows the relationship between solution temperature and set temperature of the wet digestion system. The solution temperature reached 90℃ at a set temperature of 180℃. Above these values, the set temperature was changed by 20℃ increments and boiling commenced at a set temperature of 220℃. At a set temperature of 200℃, the solution temperature was 96℃, and it was found that wet digestion could be performed without boiling the sample to avoid cross contamination. The optimum set temperature of the wet digestion system was therefore determined to be 200 ℃.

4.2. Evaluation of evaporation time (Study 2)

At 12.5 h of elapsed time from the start of heating, test

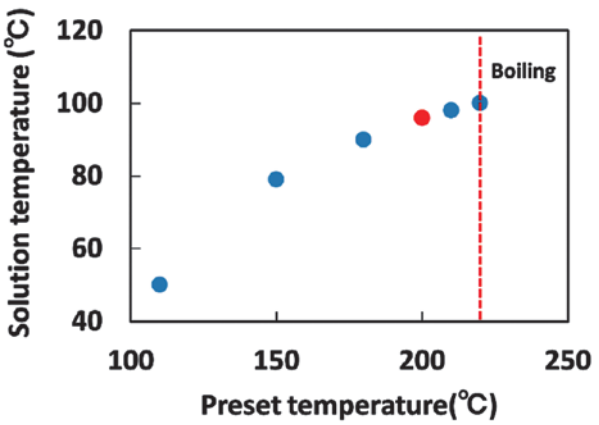

Fig. 3. Sample solution temperature vs set temperature in the wet digestion system.

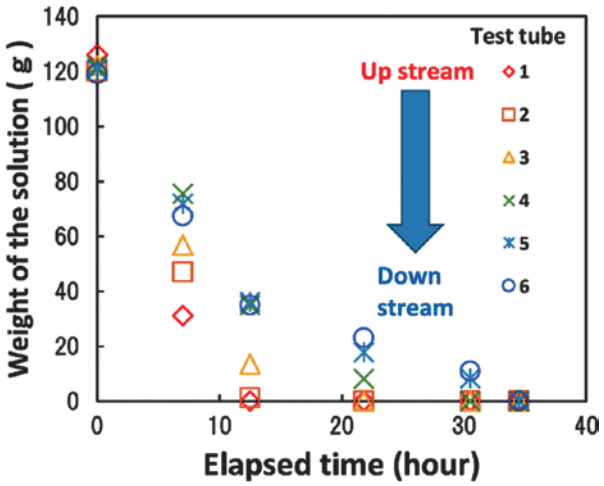

Fig. 4. Evaporation time of sample solutions at a set temperature 200℃.

Table 4. Concentration and content of ^{232}Th and ^{238}U in the orange juice

	Weight of processed sample /g	Concentration of stock solution ppt	Content /ng
^{232}Th	52.44	26 ± 5	0.87
^{238}U		111 ± 11	3.66

Table 5. Analytical results of ^{232}Th amount, recovery, cross contamination in X-STC-13 standard solution samples and blank samples of orange juice

Number of test tube	Weight of processed sample /g	Addition amount of X-STC-13 /μg	Content /ng	Content in the orange juice /ng	Net content /ng	Recovery	Contamination
1	53.53	10.4	8,060	0.42	8,060	80%	
2	49.52	-	4	0.48	3		0.04%
3	55.49	10.5	9,170	0.51	9,170	91%	
4	51.54	-	11	0.54	11		0.12%
5	49.33	10.6	8,880	0.50	8,880	88%	
6	50.99	-	16	0.50	15		0.17%

Table 6. Analytical results of ^{238}U amount, recovery, cross contamination in X-STC-13 standard solution samples and blank samples of orange juice

Number of test tube	Weight of processed sample /g	Addition amount of X-STC-13 /μg	Content /ng	Content in the orange juice /ng	Net content /ng	Recovery	Contamination
1	53.53	10.3	11,190	1.7	11,190	111%	
2	49.52	-	9	2.0	7		0.06%
3	55.49	10.4	11,820	2.1	11,800	117%	
4	51.54	-	17	2.3	15		0.13%
5	49.33	10.5	10,432	2.1	10,430	103%	
6	50.99	-	23	2.1	20		0.20%

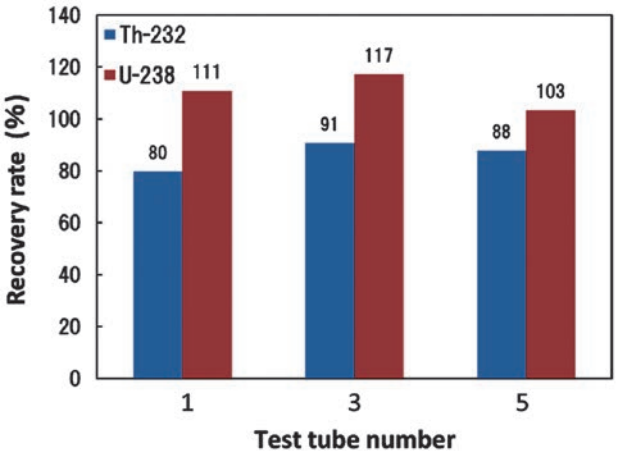

Fig. 5. Recovery of ^{232}Th and ^{238}U.

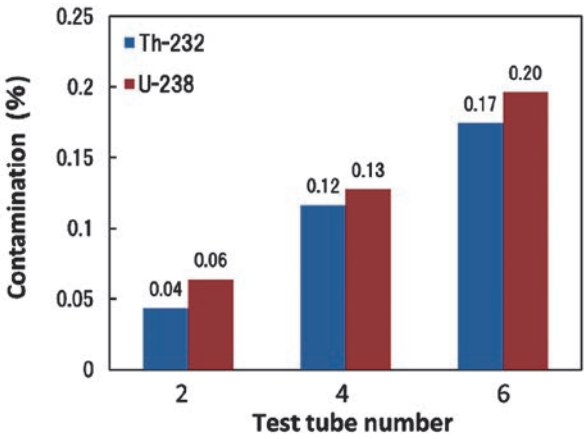

Fig. 6. Cross contamination of ^{232}Th and ^{238}U.

Table 7. Results for the simulated urine samples

Sample ID	Spiked conc./ppt	Measured conc. /ppt ^{232}Th	*Diff.*	^{238}U	*Diff.*
Urine-0	0	< 0.1		< 0.1	-
Urine-1	8.0	10.5 ± 1.6	+ 32.6%	8.0 ± 0.8	+ 0.5%
Urine-2	23.2	23.1 ± 3.0	+ 5.3%	21.8 ± 2.4	- 0.5%
Urine-3	49.6	50.0 ± 4.6	+ 0.5%	46.2 ± 4.5	- 7.3%
Urine-4	99.6	97.9 ± 4.3	- 0.7%	103.0 ± 4.8	+ 4.5%

tubes 1 and 2 on the upstream side of the exhaust system were dry. The solution in test tube 3 and 4-6 remained 13.5 g and approximately 35 g, respectively (Fig. 4). Subsequently, the evaporation time to dryness was 21.8, 30.5, 34.5 and 34.5 for test tubes 3, 4, 5 and 6, respectively. Hence, a large difference in evaporation rate depending on the position of the test tube was found and evaporation to dryness progresses from the upstream side to the downstream outlet side of the system.

4.3. Evaluation of cross contamination (Study 3)

The concentration of uranium was about 4 times higher than that for thorium (Table 4). Evaluation of recovery rate and cross contamination was conducted with X-STC-13 standard solution corrected obtained from results of orange juice alone (Tables 5 and 6). Test tube 3 had the highest recovery ratio for both thorium and uranium from test tubes 1, 3 and 5. The recovery of uranium was 100% or more for each of test tubes 1, 3, and 5 (Fig. 5). The cross contamination in blank samples was higher for uranium than thorium, and cross contamination of up to 0.20% was observed. The cross contamination tended to become higher toward the downstream side of the exhaust system (Fig. 6). Therefore, cross contamination due to droplet formation may have occurred, and needs to be considered when analyzing unknown samples.

4.4 Analysis results of simulated urine samples

The results from simulated urine samples are shown in Table 7. Measured thorium and uranium concentrations were in agreement with the spiked concentrations, within the analytical error. Only measured Thorium concentration of Urine-1 (10.5 ± 1.6 ppt) was larger than that of the spiked sample (8.0 ppt). Thorium and uranium concentrations for the blank (Urine-0) were negligibly lower than that of the spiked sample and typical urine.

4.5 Analysis results of water and urine samples

The concentration of ^{232}Th in some water samples was below the detection limit (0.03 ppt), and the maximum was 0.99 ± 0.05 ppt (mineral water b). The lowest ^{238}U concentration was 0.34 ± 0.04 ppt (tap water) with a large difference compared with the highest observed concentration of 1766 ± 12 ppt (mineral water c) (Table 8).

Figure 7 shows the progress of wet digestion of urine samples. As in Study 2, evaporation advanced from the upstream side. After 30 h, the sample became colorless and transparent owing to the digestion of organic matter (Fig. 7-b). At this time, test tubes 1 to 3 were evaporated

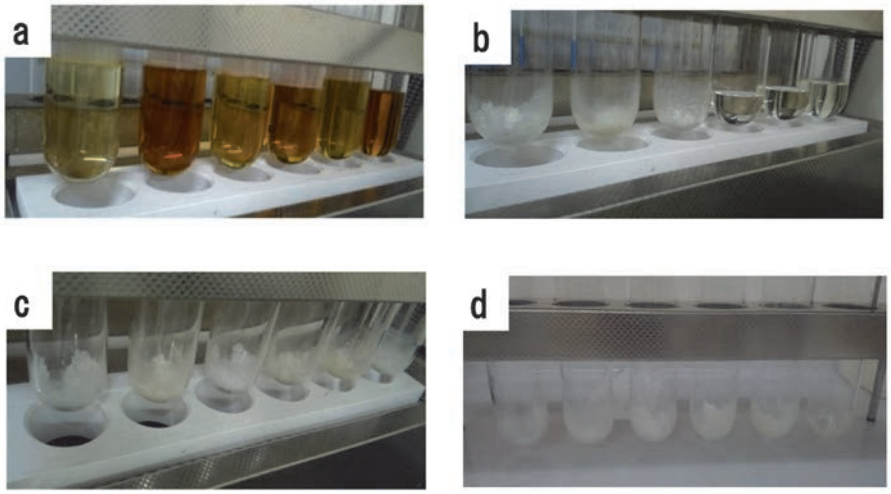

Fig. 7. Change in appearance of urine samples during wet digestion
a. Before heating
b. After heating for 30 h
c. After evaporation to dryness (after heating for 88 h)
d. After complete digestion of organic matter (after heating for 101 h)

Table 8. Analytical results of ^{232}Th and ^{238}U concentration and activities

Sample	^{232}Th		^{238}U	
	Concentration /ppt	Specific activity /nBq g^{-1}	Concentration /ppt	Specific activity /nBq g^{-1}
Tap water	0.100 ± 0.0051	0.407 ± 0.021	0.34 ± 0.04	4.2 ± 0.4
Mineral water				
a	< 0.03	0.11	6.6 ± 0.1	81.8 ± 0.9
b	0.99 ± 0.05	4.0 ± 0.2	4.5 ± 0.1	56 ± 2
c	0.24 ± 0.03	1.0 ± 0.1	1,766 ± 12	21,975 ± 154
d	0.12 ± 0.02	0.50 ± 0.08	3.47 ± 0.05	43.2 ± 0.6
e	0.14 ± 0.02	0.55 ± 0.10	0.9 ± 0.1	11.0 ± 1.0
f	< 0.03	0.11	46 ± 1	566 ± 17

Table 9. Analytical results of ^{232}Th and ^{238}U concentration and specific activities in urine samples

Sample	Test tube	Weight /g	^{232}Th		^{238}U	
			Concentration /ppt	Specific activity /nBq g^{-1}	Concentration /ppt	Specific activity /nBq g^{-1}
U4	1	52.09	43.1 ± 0.8	175 ± 3	133.2 ± 6.3	527 ± 26
U7	2	84.04	9.1 ± 0.8	37 ± 3	55.8 ± 3.2	226 ± 13
U8	3	58.73	10.7 ± 0.9	43 ± 4	35.4 ± 1.8	144 ± 7
U9	4	84.34	4.8 ± 0.6	20 ± 2	25.8 ± 1.8	105 ± 7
U10	5	82.61	12.3 ± 1.0	50 ± 4	51.1 ± 1.9	207 ± 8
U11	6	73.69	7.1 ± 1.1	29 ± 5	26.8 ± 1.8	109 ± 7

to dryness and it was considered that the other samples had decreased in volume sufficiently to allow addition of the remaining samples. The remaining samples and the same corresponding amounts of nitric acid were added and heating was continued. Evaporation was completed at 88 h (Fig. 7-c). The residues were pale yellow in test tubes 2, 4 and 5. The dry matter after digestion of organic material using an additional 5 mL of nitric acid became whitish (Fig. 7-d). Therefore, it was considered that digestion of the urine sample was complete.

The ^{232}Th concentration in the urine sample was 4.8 to 43.1 ppt and the ^{238}U concentration was 25.8 to 133.2 ppt. There was no large difference between the samples as observed for water; however, higher values were obtained for both ^{232}Th and ^{238}U concentrations than for the water samples (Table 9). This difference is thought

to result from the combination of intake from drinking water food[4]. It is reported that ^{238}U concentrations show large regional differences in Japanese mineral waters[3]. Regional differences are also reflected in the ^{238}U concentrations of the tap water samples of drinking water. In this study, the ^{238}U concentration in drinking water in Fukushima prefecture is thought to be reflected in the ^{238}U concentrations in urine.

5. Conclusion

In this study, it took about 100 h (ca. 4 days) to pretreat the urine sample (100 mL) and 1 h to measure the sample using ICP-MS by using our protocol. Therefore, it was found that it takes at least 5 days to evaluate the internal exposure dose using this protocol and approximately one week in total, taking into consideration sample collection and transport. In summary, a pretreatment method for the detection of thorium and uranium by bioassay of urine samples was established for internal exposure dose evaluation.

Conflict of Interest Disclosure

The authors declare that they have no conflict of interest.

References

1. ICRP. Individual Monitoring for Internal Exposure of Workers (preface and glossary missing), ICRP Publication 78. Ann ICRP 27. Elsevier Health Sciences; 1998.
2. Kurttio P, Auvinen A, Salonen L, Saha H, Pekkanen J, Makelainen I, *et al*. Renal Effects of Uranium in Drinking Water. Environ Health Perspect. 2002;110(4):337–42.
3. Maruyama S, Hattori K and Hirata T. Concentrations of uranium and thorium in bottled mineral waters. Chikyu Kagaku. 2014;48(3):187–99. Japanese.
4. Ota T, Sanada T, Kashiwara Y, Morimoto T and Sato K. Evaluation for committed effective dose due to dietary foods by the intake for Japanese adults. Jpn J Health Phys. 2009;44(1):80–8.
5. Nishihara K, Iwamoto H and Suyama K. Estimation of Fuel Compositions in Fukushima-Daiichi Nuclear Power Plant. No. JAEA-Data/Code. 2012-018. JAEA.2012. Japanese.
6. Shibahara Y, Kubota T, Fujii T, Fukutani S, Ohta T, Takamiya K, *et al*. 235U/238U Isotopic ratio in plant samples from Fukushima Prefecture. J Radioanal Nucl Chem. 2015;303(2):1421–4.
7. Shi Y, Collins R, Broome C. Determination of uranium, thorium and plutonium isotopes by ICP-MS. JRNC. 2013:296(1):509–15.