

Chemical Speciation of Arsenic Species in Human Blood Serum by Liquid Chromatography Using a Phosphatidylcholine-Coated ODS Column with Detection by ICP-MS

Takuya Hasegawa,[1] Jotaro Ishise,[1] Yasuharu Fukumoto,[1] Hirotaka Matsuura,[1,†] Yanbei Zhu,[1] Tomonari Umemura,[1] Hiroki Haraguchi,*[1] Kazuhito Yamamoto,[2] and Tomoki Naoe[2]

[1]Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603

[2]Department of Hematology and Oncology, Graduate School of Medicine, Nagoya University, 65 Tsurumaicho, Showa-ku, Nagoya 466-8550

Received May 10, 2006; E-mail: haraguch@apchem.nagoya-u.ac.jp

Chemical speciation of arsenic species in human blood serum was performed by high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS) with direct sample injection, where an octadecylsilyl silica (ODS) column coated with phosphatidylcholine (PC) (hereafter known as "PC-coated ODS column") was used as the separation column. In arsenic species analysis, a citrate buffer solution (pH 4.0) was used as the mobile phase, in which the following reagents were added: sodium 1-dodecanesulfonate (SDS), tetramethylammonium hydroxide (TMAH), which are ion-pair reagents to separate inorganic and organic arsenic species, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), which is a protein-solubilizing agent to prevent adsorption of proteins on the column. As a result of optimization, five representative arsenic species spiked in human blood serum reference material could be separated from each other within 5 min on the PC-coated ODS column by elution with a 5 mM citrate buffer (pH 4.0) containing 5 mM SDS, 5 mM THAH, and 0.2 mM CHAPS. The detection limits obtained by ICP-MS were 3.1, 2.7, 4.5, 2.5, and 2.5 ng of As g^{-1} for arsenate (iAs^{V}), arsenite (iAs^{III}), monomethylarsonic acid (MMA), dimethylarsenic acid (DMA), and arsenobetaine (AB), respectively, when the injection volume was 20 μL. The present separation system was also applied to speciation analysis of arsenic species in human blood serum collected from a leukemia patient after therapeutic treatment with arsenic.

In metallomics research, which was proposed as integrated biometal science,[1] it has been stressed that chemical speciation analysis for the identification and quantification of chemical species is extremely important to elucidate biological functions of the elements in a biological system.[1,2] In this regard,[1] chemical speciation of the elements in human blood, of course, is an interesting and important subject, because the accumulation of trace elements in human blood due to the food chain or occupational exposure often causes serious diseases.[3,4] In the case of arsenic, inorganic arsenic compounds such as arsenate and arsenite are known to cause acute toxicity as well as cancer. Thus, chronic exposure to them may result in several dose-related health infections.[5] On the other hand, arsenic is also known as a biologically essential element for mammals.[6]

Recently, the present authors have developed the phosphatidylcholine (PC)-coated ODS column,[7] which allowed rapid separation of large molecules, e.g., proteins, and small molecules/ions. The PC-coated ODS column was prepared by using a dynamic coating method, in which PC was hydrophobically adsorbed onto the ODS column. The PC-coated ODS column worked as a restricted access-type stationary phase for HPLC, which allowed us to do direct sample injection analysis of drugs in model blood serum samples without any pretreatment,[7] because protein adsorption was low (up to ca. 1%) on this column.

In the present experiment, chemical speciation analysis of representative arsenic species in human blood serum was examined by using HPLC with the PC-coated ODS column. In this work, arsenate (iAs^{V}), arsenite (iAs^{III}), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AB) were the species of interest. In the present arsenic species analysis, a citrate buffer (pH 4.0) containing sodium 1-dodecanesulfonate (SDS), tetramethylammonium hydroxide (TMAH), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) was used as the mobile phase to separate inorganic and organic arsenic species in ion-pair separation mode. It was essential to add CHAPS into the ion-pair reagent to prevent adsorption of proteins on the PC-coated ODS column. All of the arsenic species in blood serum were well separated from each other on the PC-coated ODS column without any deterioration of the column efficiency.

Experimental

Apparatus. The HPLC system used in the present experiment consisted of an HPLC pump (model PU-980, Jasco, Tokyo, Japan), a sample injector (model 7725, Rheodyne, Cotati, CA,

† Present address: Department of Applied Chemistry and Biochemistry, Kumamoto University, 39-1 Kurokami 2-chome, Kumamoto 860-8555

Published on the web March 13, 2007; doi:10.1246/bcsj.80.498

U.S.A.) with a 20 μL sample loop, and a UV absorption detector (model UV-970, Jasco). An ODS column (Mightysil RP-18 GP Aqua packed with 5 μm C_{18}-bonded silica, 4.6 mm i.d. × 250 mm long) was purchased from Kanto Chemicals (Tokyo, Japan). An ICP-MS instrument (model Agilent HP 4500, Yokogawa Analytical Systems, Tokyo, Japan) was used for arsenic detection (^{75}As) in HPLC. The effluent from HPLC was led to the nebulizer in the ICP-MS instrument through a Teflon tubing after the UV absorption detector.

Reagents. 3-*s*,*n*-Phosphatidylcholine, derived from egg yolk, was purchased from Wako Pure Chemicals (Osaka, Japan). Citric acid used for the mobile phase was also purchased from Wako Pure Chemicals. Ultrapure grade TMAH was obtained from Tama Chemicals (Tokyo, Japan), and tetraethylammonium hydroxide (TEAH), tetrapropylammonium hydroxide (TPAH), tetrabutylammonium hydroxide (TBAH), sodium 1-butanesulfonate (SBS), sodium 1-octanesulfonate (SOS) and SDS of chromatography grade were purchased from Tokyo Kasei Chemicals (Tokyo, Japan). All of the arsenic standard compounds were purchased from Tri Chemical Laboratories (Yamanashi, Japan). Human blood serum certified reference material, "Seronorm™ Human," was purchased from Sero As (Billingstad, Norway). All of the chemicals were used without further purification. Pure water was prepared with a Milli-Q deionization system (model Element A-10, Nihon Millipore Kogyo, Tokyo, Japan).

Preparation of the PC-Coated ODS Column. The PC-coated ODS column was prepared by using the following dynamic coating method, as described previously.[7] First, 13 mM phosphatidylcholine in methanol–water (50:50, v/v) was passed through an ODS column for 120 min at the flow rate of 0.30 mL min^{-1}, and then, the column was washed with pure water for ca. 60 min at the flow rate of 0.75 mL min^{-1}. The coating of the ODS column with PC was monitored by measuring the breakthrough curve at 210 nm.[7]

Results and Discussion

Separation of Standard Arsenic Species by Ion-Pair HPLC/ICP-MS. Inorganic arsenic species, such as iAs^{V} and iAs^{III}, and organic arsenic species, such as MMA, DMA, and AB, had the same retention times (3.50 and 3.80 min, respectively), when they were analyzed by HPLC with a PC-coated ODS column using pure water as the mobile phase.[7] In general, inorganic and organic arsenic species are analyzed by reversed-phase HPLC in ion-pair mode,[8,9] because it is possible to separate neutral, anionic and cationic species on a single column. In fact, the PC-coated ODS column has been used to separate inorganic and organic arsenic species in human urine, when HPLC was operated in ion-pair mode.[7] In the present experiment, thus, it was also operated in an ion-pair mode for arsenic analysis.

The selection of ion-pair reagents is generally crucial in ion-pair chromatography to obtain the optimum separation within the short time as much as possible. In the present experiment, a series of ion-pair reagents with different carbon lengths were examined to find the optimum separation conditions for inorganic and organic arsenic species. Tetraalkylammonium hydroxides, such as TMAH, TEAH, TPAH, and TBAH, were examined as basic ion-pair reagents, which were added into the mobile phase containing 5 mM citric acid (pH 4.0) and 5 mM SDS. The hydrophobicity of these tetraalkylammonium hydroxides increases from TMAH (one carbon in the chain) to TBAH (four carbons in the chain). Consequently, the interaction of these ion-pair reagents with the stationary phase may also increase in proportion to their carbon lengths. The experimental results for five arsenic species are summarized in Fig. 1. Form Fig. 1, clear separation of these arsenic species was achieved, when TMAH was used as the basic ion-pair reagent. On the other hand, some of arsenic species were co-eluted, when other ion-pair reagents were used. Thus, TMAH was used as an ion-pair reagent in the following experiment.

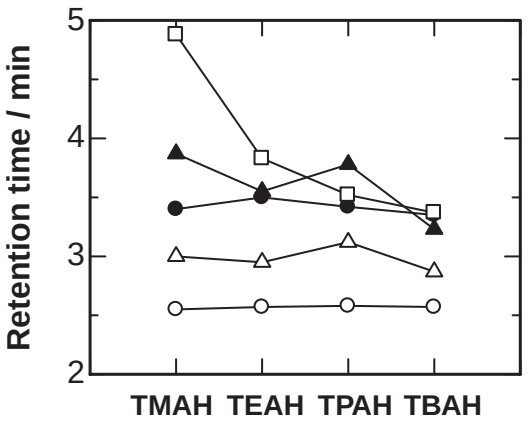

Fig. 1. Dependences of retention times of arsenic species on tetraalkylammonium hydroxides as ion-pair reagents, obtained with the PC-coated ODS column. ○ iAs^{V}, △ MMA, ● iAs^{III}, ▲ DMA, □ AB. Mobile phase, 5 mM citric acid + 5 mM SDS and 5 mM tetraalkylammonium hydroxide (pH 4.0); flow rate, 0.75 mL min^{-1}; sample injection volume, 20 μL.

Next, the effects of sodium alkylsulfonates, such as SBS, SOS, and SDS, on arsenic separation were also examined by adding them to the mobile phase containing 5 mM citric acid (pH 4.0) and 5 mM TMAH. The experimental results for the effects of these ion-pair reagents on the separation of five arsenic species are shown in Fig. 2. As can be seen in Fig. 2, when SDS was used as an acidic ion-pair reagent, better separation of arsenic species was achieved, while iAs^{III} and iAs^{V} were co-eluted in the case of SBS and SOS. These experimental results can be explained by the hydrophobicities and resultant interactions of these alkylsulfonate compounds with the stationary phase.

Furthermore, the separation of arsenic species in the ion-pair mode may be influenced by the pH conditions, because arsenic species may exist in the different chemical forms at different pH values. Thus, the effect of the pH of the mobile phase on the retention times of the arsenic species was also examined. The pH of the mobile phase (5 mM citric acid) containing 5 mM TMAH and 5 mM SDS was adjusted by adding appropriate amounts of nitric acid. The experimental results for the effect of pH on the separation of five arsenic species are shown in Fig. 3. As is seen in Fig. 3, better separation of arsenic species was achieved when the pH of the mobile phase was 4.0. At other pH values, some of the arsenic species were co-eluted.

As a result, the mixed solution of arsenic standards with the concentration of 10 ng of As g^{-1} of each compound was analyzed by using ion-pair HPLC with the PC-coated ODS col-

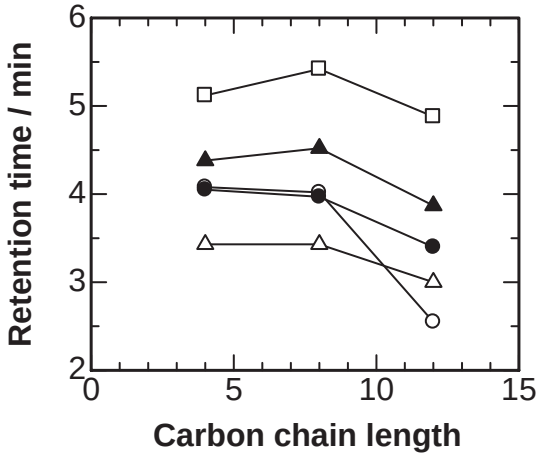

Fig. 2. Dependences of retention times of arsenic species on sodium alkylsulfonate, obtained with the PC-coated ODS column. ○ iAs^V, △ MMA, ● iAs^{III}, ▲ DMA, □ AB. Mobile phase, 5 mM citric acid containing 5 mM sodium alkylsulfonate and 5 mM TMAH (pH 4.0); flow rate, 0.75 mL min^{-1}; sample injection volume, 20 μL.

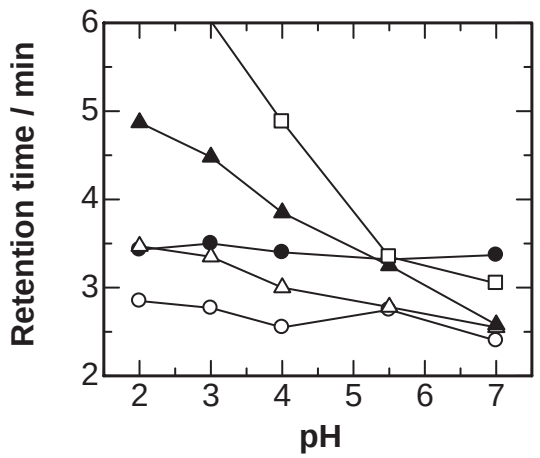

Fig. 3. Dependences of retention times of arsenic species on pH of mobile phase, obtained with the PC-coated ODS column. ○ iAs^V, △ MMA, ● iAs^{III}, ▲ DMA, □ AB. Column, PC-coated ODS column; mobile phase, 5 mM citric acid containing 5 mM SDS and 5 mM TMAH; flow rate, 0.75 mL min^{-1}; sample injection volume, 20 μL.

umn and detecting As at m/z 75 by ICP-MS. The typical chromatogram for arsenic species is shown in Fig. 4, which was obtained by using a citric acid buffer solution containing TMAH and SDS as the ion-pair reagents. As can be seen in Fig. 4, the standard arsenic compounds could be separated from each other within 6 min.

Separation of Arsenic Species in Model Blood Serum. The proposed ion-pair HPLC using a PC-coated ODS column as the stationary phase was applied to arsenic species analysis in human blood serum. A solution containing protein (3% of albumin) and arsenic compounds (10 ng of As g^{-1} in each of iAs^V, iAs^{III}, MMA, DMA, and AB) was prepared as a model serum sample and subjected to analysis. The chromatogram obtained with ICP-MS detection of As at m/z 75 is shown in Fig. 5a, along with the chromatogram with UV absorption detection (dotted line) to indicate the elution position of albumin. As can be seen in Fig. 5a, arsenic compounds in the 3% albumin solution were to some extent separated, but not well baseline-separated. Furthermore, the peak separation became

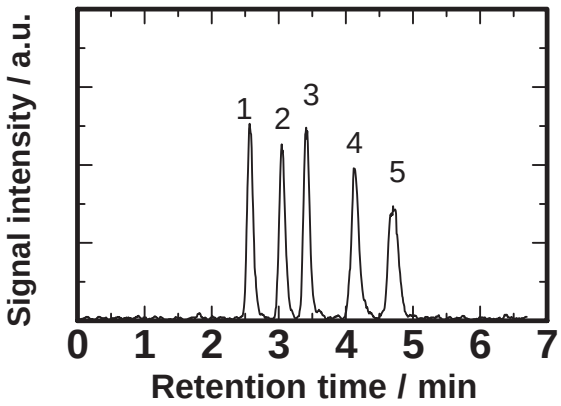

Fig. 4. Chromatogram for standard arsenic compounds obtained by ion-pair HPLC using the PC-coated ODS column with As detection by ICP-MS at m/z 75. Peaks: (1) iAs^V, (2) MMA, (3) iAs^{III}, (4) DMA, and (5) AB. Concentration, 10 ng of As g^{-1} for each compound; mobile phase, 5 mM citric acid containing 5 mM SDS, and 5 mM TMAH (pH 4.0); flow rate, 0.75 mL min^{-1}; sample injection volume, 20 μL.

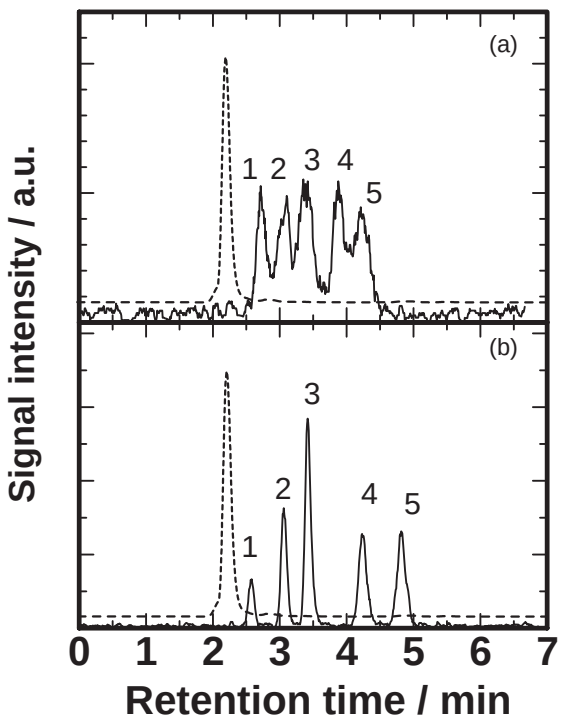

Fig. 5. Chromatograms for arsenic species in model blood serum with As detection by ICP-MS at m/z 75 (solid line) and UV absorption detection at 254 nm (dotted line). (a) Mobile phase without CHAPS, (b) mobile phase with CHAPS (0.2 mM); Model blood serum, 3% albumin aqueous solution; concentration, 10 ng of As g^{-1} for each compound. Chromatographic conditions were the same as described in Fig. 4.

Table 1. Analytical Results for 50 Repetition of the Injection of Arsenic Compounds Spiked in 3% Albumin Solution, Obtained by HPLC Using a PC-Coated ODS Column with ICP-MS Detection

	Retention time[a)]		Recovery	
	Observed value[b)] /min	RSD /%	Observed value[b)] /%	RSD /%
iAs^V	2.49 ± 0.01	0.4	117 ± 2	2
MMA	2.84 ± 0.02	0.7	102 ± 8	8
iAs^{III}	3.27 ± 0.01	0.3	94 ± 5	6
DMA	4.01 ± 0.04	1	97 ± 6	6
AB	4.82 ± 0.07	1	106 ± 7	6

a) Mobile phase: 5 mM citric acid (pH 4.0) containing 5 mM TMAH, 5 mM SDS, and 0.2 mM CHAPS. b) Mean ± S.D. ($n = 50$).

worse, when the measurements were repeated. These results suggested that the albumin adsorbed onto the surface of the PC-coated ODS column, which resulted in deterioration of separation ability of the column.

In order to improve the separation ability of the PC-coated ODS column for the protein-containing solution, 0.2 mM CHAPS was added as a protein-solubilizing agent in the mobile phase. As can be seen in Fig. 5b, arsenic species in the 3% albumin solution were baseline-separated, during which albumin was eluted at a retention time of ca. 2.1 min, as is seen as the dotted-line peak. The repeatability of the retention times and recoveries in the present system was examined by repeating the injections of the model serum solution fifty times. The experimental results for arsenic species are summarized in Table 1 together with RSDs. In addition, fairly good recoveries were also obtained for all arsenic species examined. The detection limits for iAs^V, MMA, iAs^{III}, DMA, and AB were estimated as S/N = 3 (S: peak intensity, N: baseline noise) from the chromatographic signals (peak intensity) in Fig. 5b, and they were 3.1, 4.5, 2.7, 2.5, and 2.5 ng of As g^{-1}, respectively, with ICP-MS detection (injection volume; 20 μL).

Separation of Arsenic Compounds in Human Blood Serum. Since the concentration of arsenic in human blood serum is generally very low,[1] arsenic species in blood serum could not be determined directly even by the detection with ICP-MS. Thus, in order to examine an applicability of the PC-coated ODS column to speciation of arsenic species in human blood serum, arsenic compounds were spiked in human blood serum reference material "Seronorm™ Human," and analyzed by the proposed HPLC method. The chromatogram for arsenic compounds-spiked sample is shown in Fig. 6a. The arsenic compounds could be clearly separated from each other within 6 min, but iAs^V provided the larger peak than other arsenic compounds. The peak corresponding to iAs^V was apparently enhanced by spectral interference due to $^{40}Ar^{35}Cl$ produced by the reaction of the Cl^- in the serum and Ar in plasma gas, because Cl^- was eluted almost at the same retention time as iAs^V in the PC-coated ODS column. The two peaks for ^{75}As and $^{40}Ar^{35}Cl$ could not be resolved using the low-resolution ICP-MS instrument equipped with a quadrupole mass spectrometer.

Finally, the present HPLC system using the PC-coated ODS column was used to analyze of arsenic species in human blood

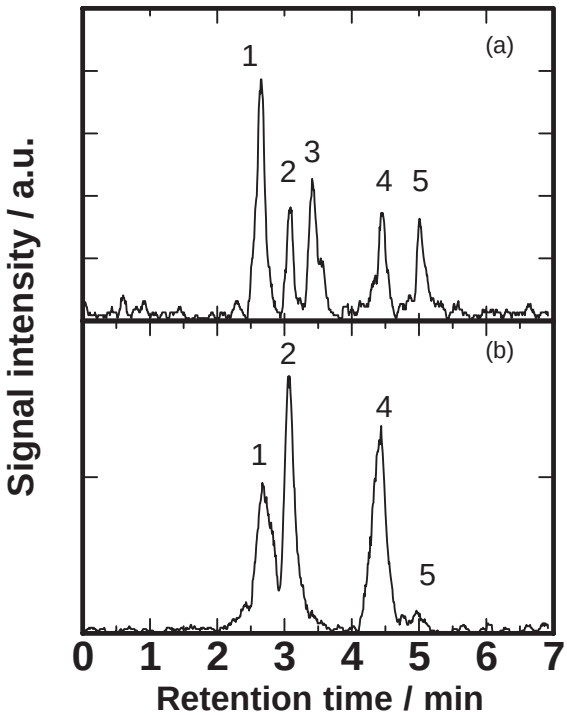

Fig. 6. Chromatograms for (a) human blood serum reference material spiked with arsenic species, and (b) human blood serum from a leukemia patient after therapeutic treatment with arsenite. Peaks: (1) iAs^V, (2) MMA, (3) iAs^{III}, (4) DMA, and (5) AB. Chromatographic conditions were the same as in Fig. 5b.

serum obtained from a leukemia patient treated with arsenite, which has recently become a therapeutic drug for leukemia. The chromatogram for arsenic compounds in human blood serum is shown in Fig. 6b. The large peaks for MMA and DMA as well as a small peak for AB were observed. The concentrations of MMA, DMA, and AB in blood serum from a leukemia patient with arsenite treatment were roughly estimated to be 12, 20, and 2 ppb, respectively, from a comparison of the peak intensities in Fig. 5b and Fig. 6b. These results suggested that arsenite (iAs^V) dosed into the vein for therapeutic treatment of leukemia was converted to methylayed arsenic compounds, such as MMA and DMA in blood. It should be noted here that the peak of iAs^V was overlapped with the interfering species of $^{40}Ar^{35}Cl$, as mentioned above, and therefore, a quantitative discussion on arsenic species distributions is difficult by the arsenic detection with low-resolution ICP-MS instrument. Thus, the arsenic detection with high-resolution ICP-MS is now under investigation to solve this problem.

Conclusion

The PC-coated ODS column was applied as a restricted access-type stationary phase for speciation of arsenic compounds in human blood serum. Inorganic arsenic, such as iAs^V and iAs^{III}, and organic arsenic, such as MMA, DMA, and AB, in aqueous solution could be separated when ion-pair reagents, i.e., 5 mM citric acid (pH 4.0) containing 5 mM TMAH and 5 mM SDS, were added to the mobile phase. However, arsenic species in a model serum sample containing 3% albumin could not be separated well, and in addition, separation was decreas-

ed in repeated measurements. In the present experiment, the separation problem was overcome by adding 0.2 mM CHAPS as a protein-solubilizing agent in the mobile phase, although the detection of iAs^V at m/z 75 (^{75}As) was interfered with $^{40}Ar^{35}Cl$. As a result, it was found that arsenite dosed in the vein of a leukemia patient for therapeutic treatment was converted mainly to MMA and DMA. Thus, it is expected that the proposed HPLC method using the PC-coated ODS column may be used in bio-medical research on leukemia treated with arsenite.

The present research was supported by the Grant-in-Aid (No. 16002009) of Specially Promoted Research, by the Grant-in-Aid (No. 17685006) for Young Scientists (A), and by the COE Basic Formation Program "Isotopes for the Prosperous Futures" from the Ministry of Education, Culture, Sports, Science and Technology.

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