Biological monitoring of o-toluidine in urine pretreated by an enzymatic deconjugation method

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Abstract

**Objectives:** To establish an enzymatic deconjugation method to separately quantify urinary o-toluidine (OT), its six metabolites, another six chemicals present in an OT-processing plant, and one metabolite of p-toluidine, and to propose optimal urinary biological monitoring items of OT exposure.

**Methods:** Thirty-six urine samples of an OT-processing plant's workers were obtained and pretreated by an enzymatic deconjugation method employing β-glucuronidase/arylsulfatase for 3 hours at 37°C and measured by liquid chromatograph-mass spectrometry (LC-MS). An alkaline hydrolytic pretreatment and 1-chlorobutane extraction procedure was also examined as a widely used urinary OT measurement method.

**Results:** The 14 chemicals were separated by LC-MS condition set by us and 13 chemicals other than 2-chloroaniline showed satisfiable linearity and limits of determination. Standard substances of six OT metabolites decomposed after the alkaline heating. In the 36 urine samples, OT, N-(4-hydroxy-2-methylphenyl) acetamide (NHM), and 4-amino-m-cresol (ACR) accounted for approx. 90% of the total OT and OT metabolites, but inter-individual variation of the three substance excretion seemed to be wide. Time course of urinary excretion revealed that concentration of the three substances was higher 24 hours after the work shift's end rather than just after the work shift.

**Conclusions:** OT and its six metabolites can each be determined with LC-MS. The alkaline method is not so optimal for exact biological monitoring. Rather, the sum of urinary OT, NHM, and ACR measured by the enzymatic method is a better index, and "end of the workweek" is a good urine-sampling time for the biological monitoring of OT exposure.

**KEYWORDS**
biological monitoring, high pressure liquid chromatography, hydrolysis, o-Toluidine, tandem mass spectrometry, β-Glucuronidase/arylsulfatase
1 | INTRODUCTION

o-Toluidine (CAS no. 95-53-4; hereafter, “OT”) is used mainly as an intermediate agent in dye manufacturing. OT is categorized as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). The occupational exposure limit (OEL) of OT recommended by the Japan Society for Occupational Health is 1 ppm, and the threshold limit value-time-weighted average (TLV-TWA) of OT recommended by the American Conference of Governmental Industrial Hygienists is 2 ppm. The biological tolerance value (BAT) of OT in urine is 0.2 μg/L by the Deutsche Forschungsgemeinschaft (German Research Foundation).

At a pigment intermediate manufacturing plant in Japan that used OT, five of 40 workers were diagnosed with bladder cancer in 2014-2015. The plant then stopped manufacturing a product made from OT (“OT product”) for approx. 6 months. As of 2017, additional five bladder cancer cases were identified at the plant and another plant of the same company.

To investigate a relationship between OT exposure and bladder cancer, a trial manufacture OT product was conducted in 2016 under the auspices of Japan’s Ministry of Health, Labor and Welfare. During the trial, the Japan National Institute of Occupational Safety and Health (JNIOSH) performed a cross-sectional survey focusing on detailed exposure settings of OT and other chemicals. JNIOSH reported that the OT exposure concentration at the plant was far less than the TLV-TWA and the OEL, but the OT levels in the urine of some workers were higher than the BAT. JNIOSH concluded that skin absorption was likely to be the major route of OT absorption.

To assess OT exposure through the skin, it is essential to measure OT and its metabolites in urine. The major method of OT measurement in urine is an alkaline hydrolysis pretreatment method proposed by the US National Institute for Occupational Safety and Health (NIOSH) (2003). Briefly, in this method urine is hydrolyzed by a sodium hydroxide solution, extracted by 1-chlorobutane, and measured with a liquid chromatograph-electrochemical detector. When this method is used, the OT, the metabolites of OT, and substances that are changed to other chemicals by the strong alkali treatment cannot be separately determined. We conducted the present study to establish an enzymatic deconjugation method to separately quantify OT, OT metabolites and other substances, and to propose optimal urinary biological monitoring indices of OT exposure.

2 | SUBJECTS AND METHODS

This study was approved by the Ethics Committee of the School of Medicine of Keio University (approval no. 20160172).

2.1 | Samples studied

Urine samples from 12 OT-exposed workers and one office worker were collected before and during the trial period of OT product manufacturing. The urine of five workers was sampled before the trial (BT) and just after their trial shift day (AS); the urine of three workers was sampled at BT, before their trial shift day (BS) and at AS, and the urine of four workers was sampled at BT, AS and 24 hours after the shift’s end (24hAS). The urine of the office worker was collected once during his normal work hours. A total of 36 urine samples were collected. Urine of six other non-exposed volunteers was sampled to establish the enzymatic deconjugation method. All samples were kept at −80°C until the measurement.

2.2 | Chemicals determined and reagents

We attempted to separately determine OT, six OT metabolites, and six chemicals that were present at the plant. The six metabolites were N-(4-hydroxy-2-methylphenyl) acetamide (NHM, CAS no. 39495-15-3), 4-aminom-cresol (ACR, CAS no. 2835-99-6), 2-amino-m-cresol (2AC, CAS no. 2835-97-4), 3-amino-o-cresol (3AC, CAS no. 53222-92-7), 3-amino-4-methylphenol (AMP, CAS no. 2836-00-2), and o-acetotoluididine (ACT, CAS no. 120-66-1). Other OT metabolites could not be assessed because we did not obtain their standard substances. The other six chemicals that were present in the plant were one acetylated OT product (2’-methylacetocetanilide [AT], CAS no. 93-68-5) and four raw materials for manufacturing other dye intermediates: (2,4-dimethylaniline [DA], CAS no. 95-68-1), o-anisidine (ANS, CAS no. 90-04-0), aniline (ANL, CAS no. 62-53-3), 2-chloroaniline (CA, CAS no. 95-51-2), and p-toluidine (PT, CAS no. 106-49-0).

A PT metabolite, p-acetotoluididine (PAC, CAS no. 103-89-9), was added in, and thus a total of 14 substances were determined. A stable isotope of 2-toluidine-d7 (OT-d7, CAS no. 68408-22-0) was used as an internal standard (IS) for OT. High-purity reagents of these substances were purchased from professional suppliers, as follows. OT >98.0%: Tokyo Chemical Industry Co. (TCI), Tokyo. NHM: Sigma-Aldrich Rare Chemical Library, approx. >90%, Sigma-Aldrich, St. Louis, MO. ACR >98.0%, 2AC >98.0%, 3AC >98.0%, AMP >98.0%, ACT >98.0%, AT >98.0%, DA >98.0%, ANS >98.0%, CA >98.0%, PT >99.0%, PAC >98.0%: all from TCI. ANL >99.0%: FUJIFILM Wako Pure Chemical Co. (FUJIFILM Wako), Osaka, Japan. OT-d7 98%: Toronto Research Chemicals, Ontario, Canada.

β-Glucuronidase/arylsulfatase (Helix pomatia; Merck, Darmstadt, Germany) and 0.1 mol/L acetate buffer solution (pH 5.0, Nacalai Tesque, Kyoto, Japan) were purchased for the enzymatic deconjugation pretreatment. Pepsin...
(FUJIFILM Wako) and pancreatin (FUJIFILM Wako) were obtained for making artificial gastric juice and intestinal juice. Water was prepared with an ultrapure water production system (Direct-Q UV, Merck). Other reagents used were special grade or LC-MS grade.

### 2.3 Measurement of substances in urine

A liquid chromatograph (LC; Agilent 1200 Infinity LC series, Agilent Technologies, Santa Clara, CA) equipped with a triple quadrupole mass spectrometer (MS; QTRAP5500, SCIEX, Framingham, MA) was used for the LC-MS measurements. A CAPCELL PAK ADME S2 column (2.1 × 100 mm, 2 μm, Osaka Soda Co., Osaka, Japan) was used for the LC separation at a column temperature of 40°C. The mobile phase (solvent A, 0.1% formic acid aqueous solution; solvent B, 0.1% formic acid methanol solution) was used at a flow rate of 0.2 mL/min for a gradient elution as follows: 0 minute—10% B, 5 minutes—10% B, 5.5 minutes—80% B, 10.5 minutes—80% B, 11 minutes—10% B, and 15 minutes—10% B. The injection volume was 10 μL. The MS was equipped with an electrospray ion source and operated in positive mode with the ion spray voltage of 4500 V. Air was used as the nebulizing and heating gas at the pressure of 0.482 and 0.552 MPa. Nitrogen gas was used as the curtain and collision gas at the pressure of 0.276 and 0.055 MPa. The MS temperature was set at 70°C. The other MS parameters of each substance are given in Table 1.

Each chemical was dissolved in methanol at the concentration of 100 μg/mL. The standard mixture solution of the 14 chemicals was prepared by diluting them with ultrapure water at the concentration of 5000 ng/mL for each just before use. The standard series for making matrix-matched calibration curves and limits of quantitation (LOQ) were prepared by adding the standard mixture solution to mixed urine of three unexposed volunteers and adjusted to the concentrations of 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, and 910 ng/mL. The LOQs were set for the concentrations at which the signal-noise ratios on the chromatogram exceeded 10. The standard series for confirming the spike recoveries was prepared by adding the standard mixture solution to urine of the other three unexposed volunteers at the concentrations of 50, 100, and 200 ng/mL.

### 2.4 Pretreatment for the enzymatic deconjugation method

β-Glucuronidase/arylsulfatase was diluted with acetate buffer and adjusted to 4 U/mL of the specific activity of β-glucuronidase (enzyme solution). OT-d7 was diluted with an aqueous solution (methanol : water = 1 : 100, v/v) and adjusted to 100 ng/mL (IS solution). Thawed 100 μL of urine samples was mixed with 100 μL of the enzyme solution, incubated for 3 hours at 37°C under protection from light, cooled to room temperature, and added with 50 μL of a 2.5% formic acid methanol solution to stop the enzymatic reaction.

### Table 1

| Substances | Retention time (min) | MRM transitions (m/z) | MRM parameters (V) |
|------------|---------------------|-----------------------|---------------------|
|            | Precursorion | Production | Declustering potential | Entrance potential | Collision energy | Collision cell exit potential |
| OT         | 6.0       | 107.9 91.1 | 96            | 10 | 25 | 8 |
| NHM OT metabolite | 8.3 | 166 107 | 60            | 10 | 30 | 10 |
| ACR OT metabolite | 2.0 | 123.9 109.1 | 91            | 10 | 23 | 8 |
| 2AC OT metabolite | 4.6 | 124 79 | 60            | 10 | 30 | 18 |
| AMP OT metabolite | 3.5 | 124.1 76.9 | 126          | 10 | 35 | 12 |
| 3AC OT metabolite | 2.5 | 124.2 76.9 | 96            | 10 | 35 | 6 |
| ACT OT metabolite | 8.7 | 150 93.1 | 91            | 10 | 35 | 8 |
| DA raw material | 8.3 | 121.9 107.1 | 116          | 10 | 23 | 10 |
| ANS raw material | 5.4 | 123.9 65 | 56            | 10 | 33 | 8 |
| AT raw material | 8.8 | 192 108.2 | 71           | 10 | 39 | 10 |
| ANL raw material | 2.8 | 93.9 51 | 126          | 10 | 39 | 8 |
| CA raw material | 9.1 | 127.9 92.1 | 86           | 10 | 23 | 8 |
| PT raw material | 6.5 | 107.9 91.1 | 96           | 10 | 25 | 8 |
| PAC PT metabolite | 9.0 | 150.2 108.2 | 91           | 10 | 23 | 16 |
| OT-d7 internal standard | 5.6 | 115 97.7 | 101           | 10 | 25 | 8 |

Abbreviations: LC-MS, liquid chromatograph-mass spectrometry; MRM, multiple reaction monitoring.
The samples were then shaken vigorously for 15 seconds and centrifuged at 18,000 g for 5 minutes. The supernatant was applied to a Nanosep® centrifugal device with omega membrane (3K, Pall, Port Washington, NY) and centrifuged at 14,000 g for 30 minutes. Next, 100 μL of the fluid passing through was mixed with 100 μL of IS solution and measured by LC-MS. All tubes and containers used for preservation, pretreatment, and measurement were made of polypropylene, because the ACR aqueous solution easily decreases in some glass containers.

### 2.5 Stability against alkaline hydrolysis pretreatment and artificial digestive juice

For the confirmation of the stability of the six OT metabolites and seven substances against the alkaline hydrolysis pretreatment, each substance was treated according to the NIOSH alkaline hydrolysis procedure, and the 1-chlolobutane extracted layer was instilled in the LC-MS system.

Artificial gastric fluid recommended by the International Life Science Institute and artificial intestinal fluid defined by the US Pharmacopeia were applied for the estimation of whether AT transforms to OT in the human body. First, 10 μL of OT or AT aqueous solutions were added to 990 μL of artificial gastric or intestinal fluids, incubated for 1 hour at 37°C under protection from light, and the 1-chlolobutane extracted layer was instilled in the LC-MS system.

### 2.6 OT measurement by the NIOSH method

The OT concentration of the 36 workers' urine was measured at a nationwide clinical laboratory where a partially modified NIOSH alkaline hydrolysis pretreatment method was used.

### 3 RESULTS

#### 3.1 The separation, identification, and quantification performance of LC-MS

The retention peaks of DA and NHM and those of ACT and AT overlapped on the LC chromatogram, and they were discriminated by multiple reaction monitoring transitions of the mass spectrometry. The other 10 chemicals could be clearly separated by chromatography. Thirteen chemicals (CA was the exception) were sufficiently quantified. Poor sensitivity and precision were observed for CA.

The matrix-matched calibration curve of OT was plotted by the internal reference method, and the curves of the other 13 compounds were plotted by the absolute calibration method. The linear calibration curves obtained by the method of least squares were separately assessed by low (0-100 ng/mL) and high (100-910 ng/mL) concentration ranges to guarantee the accuracy at the low concentration. Except for CA, the compounds showed good linearity at the low concentration range ($r = 0.998-1.000$) as well as at the high concentration range ($r = 0.998-1.000$). The LOQs were obtained as 30-220 nmol/L; the unit was converted from ng/mL to nmol/L for the comparison of the sensitivities of the substances. And the spiked recoveries were 85%-115%. On the other hand, the validation data of the alkaline hydrolysis method were as follows: correlation coefficient of the linear calibration curve obtained by the method of least squares, 0.996; accuracy, −12.2% to 14.0% at the range of 5-1000 ng/mL; spiked recoveries, 93%–106% at the urinary concentrations of 10, 300, and 800 ng/mL; LOQ, 50 nmol/L. The details are described in Table 2.

#### 3.2 Optimization of the enzymatic reaction time

To identify the optimal incubation time for the enzymatic reaction, we incubated the urine samples of three highly exposed workers for 0, 1, 2, 3, 4, 6, and 8 hours according to the enzymatic deconjugation pretreatment procedure. Figure 1 illustrates the relationship between the incubation time and reactive products. Although there were individual differences among the three examined workers, the concentrations of OT and NHM reached the steady state 3 hours after the start of the reaction. The concentration of ACR reached its maximum at 1-3 hours after the start of the reaction and then began to decrease. The compound 2AC was detected in one of the three workers. The concentration of 2AC sharply increased 2 hours after the start of the reaction, and then maintained a slight increase. The concentrations of the other compounds (3AC, AMP, and ACT) were less than the LOQs in all three samples. Based on these data, we decided to use 3 hours for the enzymatic incubation time because the concentration of NHM, which occupied a large part of the sum of urinary OT and its metabolites, reached its maximum 3 hours after the start of the reaction.

#### 3.3 The stability of the substances

After the NIOSH alkaline hydrolysis pretreatment and 1-chlorobutane extraction procedure was applied to the 13 substances, all ACT converted to OT, and AT was severely diminished and converted to OT and ACT (Supplementary Figure S1). OT was not detected from the extracted layer of the other 11 substances. Five OT metabolites (NHM, ACR, 2AC, 3AC, and AMP) were not detected or scarcely detected from the extracted layer. We verified whether AT converted to OT in a gastrointestinal tract. After a 1-hour incubation of AT in artificial gastric or intestinal fluid at 37°C, no OT was detected. These results indicate that the OT concentration in urine measured by the NIOSH method...
is the sum of OT, ACT, partially AT, and other substances that were not identified in this study.

### 3.4 The urinary concentration of the 14 substances in the 36 samples

Table 2 shows the urinary concentrations of the 14 substances and the sum of the concentrations of OT and its six metabolites ACR, 2AC, 3AC, AMP, NHM, and ACT (for short, 'MET7') in the 36 samples. The unit of each concentration is converted from ng/ml to nmol/L. The term 'aOT' means the OT concentration obtained by the NIOSH method. The detection rate of aOT (17/36) was smaller than that of OT (26/36) by the enzymatic deconjugation method. Of the OT metabolites, NHM and ACR were detected in 33 and 24 of the 36 samples, respectively, but ACT, AMP, and 3AC were all <LOQ.

Figure 2 illustrates the concentration and proportion of MET7 in each sample. OT and its six metabolites were not determined in one worker's BT urine, and thus there are 35 bars in the figure's graph. Almost all of the MET7 was OT (mean 20%), NHM (mean 51%), and ACR (mean 29%). However, inter-individual variation of the three chemical excretion seemed to be wide. Some substances other than OT and OT metabolites were detected in only a few of the workers' urine.

### 3.5 The time course of OT and OT metabolites

Table 3 shows the time courses of the urinary excretion of aOT, OT, NHM, ACR, and the sum of OT, NHM, and ACR (for short, MET3) by urine sampling timing. The concentration of all five indices clearly and sharply increased with time.

### 4 Discussion

By using a separation column with an adamantyl group, aminocresol isomers (which increase their water solubility by hydroxylation) can be retained by the column; the structural low-molecular-weight isomers with an aromatic ring can be separated within a reasonable retention time. Thus, under the LC-MS conditions described above, we can sufficiently separate and accurately quantify OT, six OT metabolites and other six substances (Table 2). As far as we know, this LC-MS condition is firstly reported in the world.

The concentrations of NHM, ACR, OT, and 2AC increased by an action of β-glucuronidase/arylsulfatase (Figure 1). It is speculated that, in theory, NHM (which has the hydroxyl
group) was deconjugated from O-glucuronide or sulfate; OT (which has the amino group) was deconjugated from N-glucuronide or sulfate; and aminocresol isomers (which have the hydroxyl group and the amino group) were deconjugated from O- or N-glucuronide or sulfate.9

As illustrated in Figure 2, not only OT but the OT metabolites were detected in urine measured by our enzymatic deconjugation pretreatment method with a 3 hours enzymatic incubation time. The concentration of OT was lower than those of NHM and ACR in most of the urine samples; and the proportion of OT varied widely among the urine samples. On the other hand, after the NIOSH alkali hydrolysis pretreatment and 1-chlorobutane extraction procedure, six OT metabolites were scarcely or not detected. This indicates that, if urinary OT is used as an OT exposure index, the OT measured by the NOISH method leads to an over- or under-estimation of the OT exposure level through the skin and/or via inhalation. Additionally, if the OT metabolites such as
NHM and ACR (which have larger proportions than OT) are included in the OT exposure index, lower exposure cannot be missed.

Because NHM and ACR are the major OT metabolites observed in urine in this survey, other metabolites in urine were at practically negligible levels, and the proportion of these two metabolites was approx. 80%, we propose that the sum of OT, NHM, and ACR is a reasonable OT biological monitoring index in urine as determined by our enzymatic deconjugation pretreatment procedure for a 3-hour incubation and the LC-MS measurement conditions shown above. A study of subcutaneous OT administration in rats showed that 59.6% of the loading 14C dose was excreted in urine 24 hours after the administration, and the major metabolites were OT, conjugated/unconjugated ACR, and conjugated/unconjugated NHM, and the respective proportions (%) of these substances in urine were 8.6 (5.1/59.6), 51.2(30.5/59.6), and 19.5 (11.6/59.6). These results are not contradictory to our present findings.

In our study, the concentrations of OT, ACR, and NHM were higher in urine collected 24 hours after the workers' shift than in urine collected immediately after the shift. However, in a study using a rat model, the metabolites (conjugates of ACR) showed slower excretion and the unchanged OT showed relatively rapid excretion. The 24hAS OT value may thus be affected by the current day's OT exposure. Concerning NHM and ACR of the metabolites, a substantial portion of these two compounds did not reach the phase of urinary excretion just after the shift, and the urinary excretion of these compounds increased after that. Therefore, the optimal urine sampling time for the biological monitoring of OT exposure is not "after the shift on a work day" but "after the shift of the last day of a successive shift."

NIOSH states that "biological monitoring using pre and post shift urine samples is recommended in order to capture the metabolites quickly after exposure." This is not compatible with our suggestion. The reason for this discrepancy in recommended sampling times may be due to the difference in the substances referred to by NIOSH and those examined by our research group. Although both NIOSH and we measure OT and its metabolite(s), NIOSH measures acetyl metabolite (which is presumed to be ACT) and monitors mainly OT. Our measurements revealed much less ACT than OT; actually, ACT was less than the LOQ in all of the urine samples. In addition, we focused on two other metabolites, NHM and ACR, and we observed that their levels were considerably higher than those of OT. Our recommended index consisting of OT, NHM, and ACR is thus strongly affected by the rates of generation and elimination of the metabolites, and the optimal sampling time delays as a result.

We detected substantial concentrations of OT and some OT metabolites in the urine sampled before the trial. In their study conducted at a tire manufacturing plant, Stettler et

| Table 3 | Concentrations of OT, NHM, ACR, and the sum of OT, NHM, and ACR (MET3) by sampling timing |
|---------|---------------------------------|
| **OT**  | **NHM** | **ACR** | **MET3** |
| BT      | AS     | 24hAS  | BT      | AS     | 24hAS  |
| AM      | <LOQ   | <LOQ   | <LOQ    | <LOQ   | <LOQ   |
| GM      | 74     | 517    | 52      | 579    | 7006   |
| GSD     | 2.3    | 3.0    | 3.9     | 4.8    | 4.4    |
| MED     | <LOQ   | <LOQ   | <LOQ    | <LOQ   | <LOQ   |
| Min     | <LOQ   | <LOQ   | <LOQ    | <LOQ   | <LOQ   |
| Max     | 125    | 154    | 94      | 357    | 7006   |

Unit: nmol/L. Sample numbers of BT, AS, and 24hAS were 13, 12, and 4.

Abbreviations: <LOQ, less than the limit of quantitation; 24hAS, 24 hrs after the shift; AM, arithmetic mean; AS, just after the workers' trial shift day; BT, before the trial; GM, geometric mean; GSD, geometric standard deviation; Max, maximum; MED, median; Min, minimum.
al. observed that an arithmetic mean (AM) of the urinary OT concentration before the work shift was 173 nmol/L in exposed workers and 10 nmol/L in un-exposed workers. In their study at a chemical manufacturing plant, Ward et al. reported that the AM of the urinary OT concentration before a work shift was 144 nmol/L in exposed workers and 27 nmol/L in unexposed workers. The present study was conducted after OT disuse for approx. 6 months, and therefore the subjects would correspond to an unexposed group. Their urinary OT concentrations, which were obtained by alkaline pretreatment (as in the above two studies) were comparable to those in the Stettler et al. and Ward et al. studies: the AM of aOT before the trial was <LOQ (50 nmol/L). The IARC lists non-occupational exposure sources of OT as follows: air, water, soil, food and beverages, tobacco smoke, hair dyes, and the anesthetic prilocaine. Therefore, the detection of OT and OT metabolites in urine collected before the trial is not a measurement error.

This study has some limitations. First, no quantitative absorption data through the skin or via the respiratory tract were available, and we thus could not analyze the relationship between the OT absorption and urinary excretion. Second, though Son et al. noted that 59.6% of the applied dose of OT and its metabolites was excreted in urine by 24 hours and 83.0% by 48 hours; our urinary sampling was conducted only at 24 hours after the shift. Third, the standard substances of azoxytoluene because we could not obtain their standard substances. The urinary concentrations of these compounds are unknown. Fifth, if OT-exposed subjects are co-exposed to NHM and/or ACR or co-exposed to chemicals being metabolized to NHM and/or ACR, the MET3 index for OT excretion may be overestimated. In this study, we considered the possibility that AT converts to ACT or OT and changes to NHM or ACR in digestive juice, but we confirmed that AT is stable in digestive juice. Nevertheless, the possibility of the transformation of absorbed AT to NHM and ACR remains.

Fourth, we did not measure several OT metabolites including anthranilic acid, N-acetyl-anthranilic acid, N-acetyl-o-amino benzyl alcohol, o-nitrosotoluene, and o-azoxytoluene because we could not obtain their standard substances. The urinary concentrations of these compounds are unknown. Fifth, if OT-exposed subjects are co-exposed to NHM and/or ACR or co-exposed to chemicals being metabolized to NHM and/or ACR, the MET3 index for OT exposure may be overestimated. In this study, we considered the possibility that AT converts to ACT or OT and changes to NHM or ACR in digestive juice, but we confirmed that AT is stable in digestive juice. Nevertheless, the possibility of the transformation of absorbed AT to NHM and ACR remains.

**5 | CONCLUSIONS**

We established an LC-MS analytical method to separate and measure OT and its six metabolites simultaneously with enough accuracy and precision, and we compared a general alkaline hydrolysis pretreatment and a newly constructed enzymatic deconjugation pretreatment. The alkaline pretreatment destroys OT metabolites and more, and it thus cannot be used for exact biological monitoring. Instead, the enzymatic pretreatment deconjugates OT metabolites in urine and maintains their main structures, and they can each be measured by new LC-MS method. Consequently, we recommends the sum of urinary OT, NHM, and ACR as an index for the biological monitoring of OT exposure, and "after the shift of the last day of a successive shift" for the sampling time of urine.

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**DISCLOSURE**

Approval of the research protocol: This study was approved by the Ethics Committee of the School of Medicine of Keio University (approval no. 20160172). Informed consent: N/A. Registry and the registration no. of the study/trial: N/A. Animal studies: N/A. Conflict of interest: The authors declare that they have no competing interests.

**AUTHOR CONTRIBUTIONS**

YE conceived and practiced the measurement, and drafted the manuscript. MN coordinated the study and interpreted the data. TK checked the measurement and interpreted the data. KO interpreted the data and wrote the manuscript. TT designed and coordinated whole the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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