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Stir bar sorptive extraction with in situ de-conjugation and thermal desorption gas chromatography-mass spectrometry for measurement of 4-nonylphenol glucuronide in human urine sample

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Abstract

4-Nonylphenol glucuronide (NP-G) in human urine samples was analyzed using stir bar sorptive extraction (SBSE) with in situ de-conjugation by β-glucuronidase and thermal desorption (TD)-gas chromatography-mass spectrometry (GC-MS). Distilled water (1 ml), 1.0 M ammonium acetate solution (100 μl) and β-glucuronidase (10,000 units ml^{-1}, 10 μl) were added to human urine sample (1 ml), and extraction was commenced for 90 min at 37 °C while stirring at 250 rpm with a stir bar coated with a 500-μm-thick polydimethylsiloxane (PDMS) layer. Then, the stir bar was subjected to TD-GC-MS in the selected ion monitoring (SIM) mode. The calibration curve was made by SBSE method using 4-nonylphenol (NP) as the standard solution. The method showed good linearity and the correlation coefficients were 0.999 over the concentration range of 5–500 nM. Moreover, to optimize the conditions for SBSE with in situ de-conjugation and the recovery test, NP-G was synthesized by a biochemical technique in our laboratory. The limits of detection ($S/N = 3$) and quantitation ($S/N > 10$) for NP were 0.2 ng ml^{-1} (1.0 nM) and 1.1 ng ml^{-1} (5.0 nM), respectively. The average recoveries in the human urine samples ($n = 6$) spiked with NP-G at levels of 20 and 100 nM were 104.1 (R.S.D. 7.1%) and 100.6% (R.S.D. 9.2%), respectively, with correction using the added internal standard, 4-(1-methyl) octylphenol-d_5. The method enabled the precise determination of the standard and was applicable to the detection of trace amounts of NP-G in human urine samples.

Keywords: Stir bar sorptive extraction (SBSE); In situ de-conjugation; Urine; Nonylphenol; Thermal desorption (TD)

1. Introduction

Nonylphenolethoxylates (NPEs) comprise the major class of non-ionic surfactants and have a variety of industrial and commercial applications. 4-Nonylphenol (NP) is the degradation product of NPEs and has been shown to exist in such environments as seawater, river water and sewage wastewater [1–10]. It has been found to induce estrogenic responses in male trout [11,12] and a variety of bioassays [13–15]. Recently, the leaching and contamination of NP from food wrapping films, food-contacting plastics, toys and foods have been reported [16–18], exposing healthy humans to NP. Therefore, the risk assessment of NP is an important issue.

In a previous study of the pharmacokinetic behavior of NP, its elimination half-life from blood and bioavailability (determined from oral and intravenous area under the blood

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concentration-time curves (AUCs)) were found to be 2–3 h and 20%, respectively. The low bioavailability was due to its extensive metabolism as glucuronide during the first passage in the liver and excretion via the urine [19]. Therefore, it is possible to perform exposure assessment of NP by measuring of nonylphenol glucuronide (NP-G) in human urine sample. In this study, we show that NP-G can be measured in human urine samples. A method that involves solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) has been reported for the determination of NP in human urine samples [20]. However, because the de-conjugation of NP-G by β-glucuronidase was necessary prior to the SPE, the operation is tedious and time-consuming. Moreover, the above-mentioned analytical methods may not have sufficient sensitivity for the determination of trace amounts of NP technical isomers in human urine samples [21]. Therefore, a simple, accurate and highly sensitive analytical method is required.

Recently, a new sorptive extraction technique that uses a stir bar coated with polydimethylsiloxane (PDMS) was developed and called stir bar sorptive extraction (SBSE) [22]. Depending upon their octanol/water partition coefficients, the compounds are extracted and enriched. This technique has been applied successfully in the determination of volatiles, semivolatiles, stale-flavored carbonyl compounds, pesticides, mycotoxin, polycyclic aromatic hydrocarbons and dicarboximide fungicides in various samples [23–30]. In addition, SBSE has been applied successfully in biological samples [31–33]. We have reported the utility of SBSE for the measurement of endocrine disruptors (EDs) in human, water and animal food samples [34–43]. In addition, determination of NP and octylphenol (OP) in urine sample by SBSE method was already reported [35]. In the previous study, the de-conjugation process by enzyme and extraction process by SBSE method were separately performed. Moreover, in most of all biomedical analysis using SBSE method, they were separately performed [32,36,42,43]. Therefore, the sample preparation process is tedious and time-consuming, and they are important issue.

The aim of this study was to develop an analytical method that enables simultaneous SBSE and de-conjugation, which involves the addition of β-glucuronidase during the SBSE, and is called "SBSE with in situ de-conjugation." Then, the determination of trace amounts of NP-G in human urine samples by SBSE with in situ de-conjugation and thermal desorption (TD)-GC-MS was attempted. The SBSE with in situ de-conjugation method is expected high utility in biomedical investigation.

2. Experimental

2.1. Materials and reagents

NP technical isomers [M.W. 220] of environmental analytical grade was purchased from Kanto Chemical Inc., Tokyo, Japan. NP-G was synthesized by our laboratory as described below. 4-(1-Methyl) octylphenol-d_5 (NP-d_5) was purchased from Hayashi Pure Chemical Inc., Osaka, Japan. The chemical structures are shown in Fig. 1. NP-G was synthesized with reference to the minor modifications in previous studies [44,45]. UDP-glucuronic acid trisodium salt and sodium cholate of biochemical grade, magnesium chloride, tris(hydroxymethyl)-aminomethane hydrochloride, ammonium acetate and other organic solvents were purchased from Wako Pure Chemical Inc., Osaka, Japan. Pooled male rat liver microsomes were purchased from BD Biosciences Discovery Labware, Woburn, MA, USA. *E. coli* β-glucuronidase (25,000 units (0.4 ml)$^{-1}$) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Prior to use, the β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 units ml^{-1}. The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). The EDS polisher was a new filter purchased from Millipore, Japan.

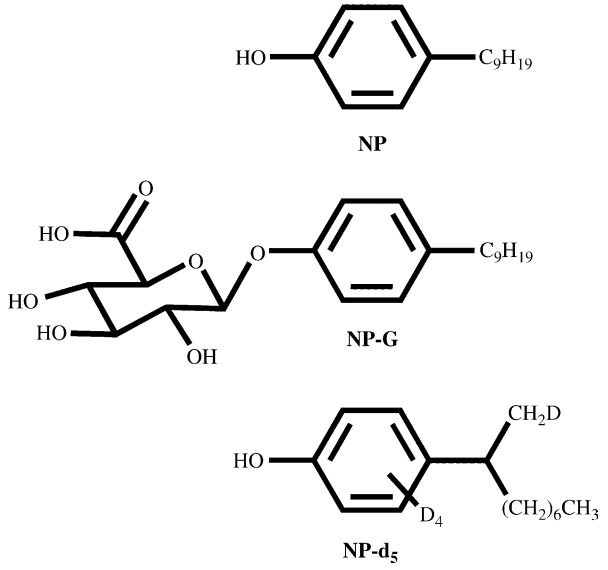

Fig. 1. Chemical structures of NP, NP-G and NP-d_5.

2.2. Standard solutions

The stock solution (1.0 mM in acetonitrile) of NP was prepared as required by the addition of purified water. Seven-point calibrations (5–500 nM for NP) were performed daily for all the samples with internal standards. The stock solution of NP-G was prepared at 50 μM in methanol.

2.3. Human urine samples

Human urine samples were collected from six healthy volunteers (22–25 years old). All samples were stored at −20 °C prior to use.

2.4. Instrumentation

Stir bars coated with 500-μm-thick (24 μl) PDMS (Twister™: the magnetic stirring rod is incorporated in a glass jacket and coated with PDMS) were obtained from Gerstel (Mullheim an der Ruhr, Germany). The stir bars were conditioned for 1 h at 300 °C in a flow of helium. Then, the stir bars were kept in new 2 ml vials until immediately prior to use. The stir bars could be used more than 50 times with appropriate re-conditioning. For the extraction, 10 ml headspace vials from Agilent Technologies (Palo Alto, CA, USA) were used. TD-GC-MS analysis was performed using a Gerstel TDS 2 thermodesorption system equipped with a Gerstel TDS-A autosampler and a Gerstel CIS 4 programmable temperature vaporization (PTV) inlet (Gerstel), and an Agilent 6890N gas chromatograph with a 5973N mass-selective detector with ultra ion source (Agilent Technologies).

2.5. TD-GC-MS conditions

The conditions of TD-GC-MS were set referring to previous study [34,35]. The temperature of TDS 2 was programmed to increase from 20 °C (held for 1 min) to 280 °C (held for 5 min) at a rate of 60 °C min^{-1}. The desorbed compounds were cryofocused in the CIS 4 at −150 °C. After desorption, the temperature of the CIS 4 was programmed to increase from −150 to 300 °C (held for 10 min) at a rate of 12 °C s^{-1} to inject the trapped compounds into the analytical column. The injection was performed in the splitless mode. Separation was accomplished on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.5 μm film thickness, Agilent Technologies). The oven temperature was programmed to increase from 60 to 300 °C (held for 5 min) at a rate of 15 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. The mass spectrometer was operated in the selected ion monitoring (SIM) mode with electron ionization (ionization voltage: 70 eV). Quantitative analysis was performed in the SIM mode in order to maximize sensitivity. Three ions were monitored for SIM (*m/z* 135, 107 for NP and *m/z* 126 for NP-d_5. The underlined number is the *m/z* of the ion used for determination.). The mass spectra of analyte are shown in Fig. 2.

2.6. SBSE with in situ de-conjugation of NP-G in human urine sample

Urine sample (1.0 ml), distilled water (1.0 ml), 1.0 M ammonium acetate solution (100 μl) and β-glucuronidase (10 μl) were placed in a glass vial. A stir bar coated with PDMS was added and the vial was crimped with a Teflon-coated silicone septum cap. SBSE of the sample was performed at 37 °C for 90 min while stirring at 250 rpm. After the extraction, the stir bar was easily removed with forceps (due to the magnetic effect), rinsed with distilled water, dried with lint-free tissue and placed in a glass TD tube. The TD tube was then placed in the TD unit. After thermally desorbing the stir bar in the TD system, GC-MS was conducted.

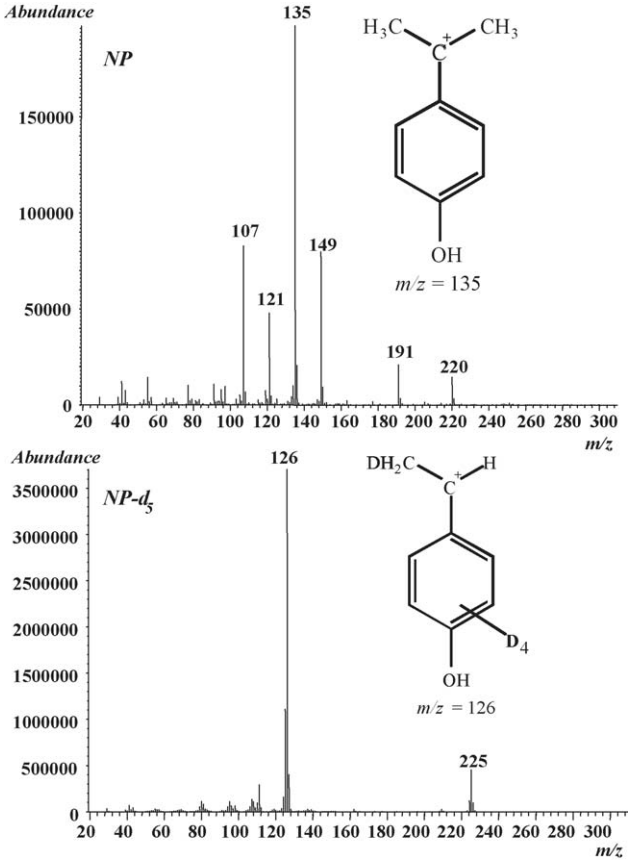

Fig. 2. Mass spectra of NP and NP-d_5.

3. Results and discussion

3.1. Theoretical recovery

Log $K_{o/w}$ and the theoretical recoveries of the compounds were investigated in this work. The log $K_{o/w}$ values were calculated with the log *P* predictor, which is available from Interactive Analysis Inc. (Bedford, MA, USA). The log $K_{o/w}$ of NP-G and NP were 0.99 and 5.96, respectively. Theoretical recovery was calculated with the following equations:

$$\text{theoretical recovery} = \frac{K_{o/w}/\beta}{1 + K_{o/w}/\beta} = \frac{1}{\beta/K_{o/w} + 1}$$

where $\beta = V_s/V_{PDMS}$, V_{PDMS} being the volume of PDMS, and V_s, the volume of sample. The theoretical recoveries by SBSE were calculated on the basis of a 2 ml sample volume and a stir bar with a phase thickness of 500 μm (24 μl of PDMS). Because the theoretical recoveries of NP-G and NP were 10.5 and 100.0%, respectively, the analysis by SBSE with in situ de-conjugation was considered to have high recovery.

3.2. Optimization of conditions for SBSE with in situ de-conjugation

The most important parameters affecting SBSE with in situ de-conjugation were the extraction time and the stirring speed. Therefore, the optimum extraction time and stirring speed were investigated using human urine samples spiked with NP-G (final concentration: 100 nM). SBSE with in situ de-conjugation of the NP-G from a human urine sample by β-glucuronidase was performed by placing a suitable amount of sample in a headspace vial. The extraction time and the stirring speed were investigated in the range of 0–150 min and 0–500 rpm, respectively. The extraction time profiles (equilibration curves) of NP in the human urine samples using the stir bar were determined by TD-GC-MS and are shown in Fig. 3 NP reached equilibrium after approximately 90 min extraction at 250 and 500 rpm. There was little difference between 250 and 500 rpm. Because

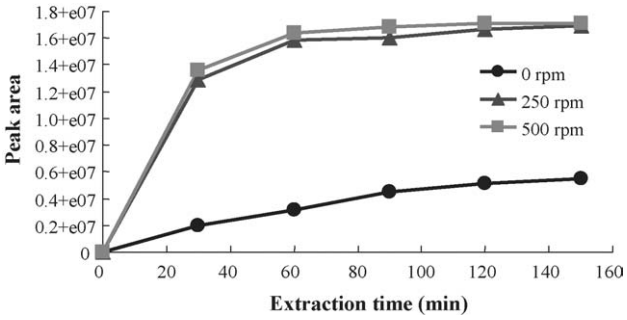

Fig. 3. Optimum extraction time and stirring speed for analysis of NP-G by SBSE with in situ de-conjugation.

the enzyme is made of the protein, the slow stirring speed was assumed to be the optimum condition. Therefore, the extraction time and the stirring speed were set at 90 min and 250 rpm, respectively. The complete de-conjugation

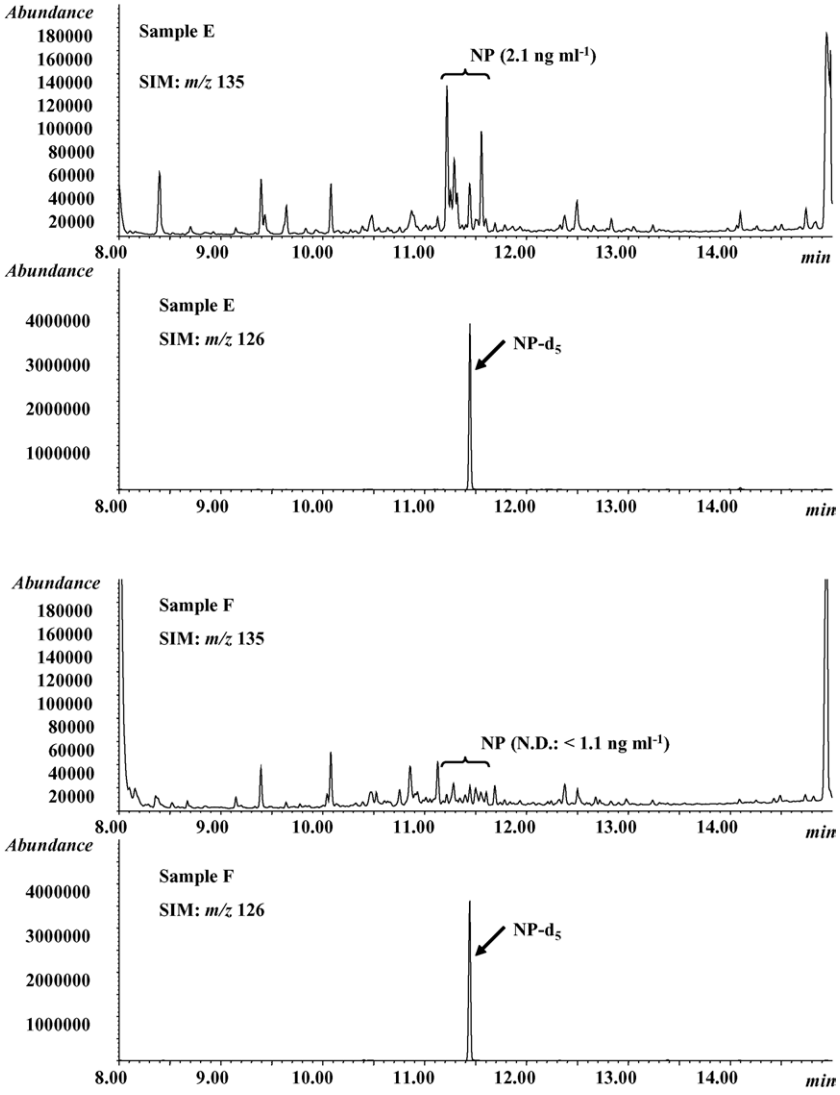

Fig. 4. SIM mass chromatograms of NP and internal standard in human urine sample (volunteer E and F) subjected to SBSE with in situ de-conjugation.

was achieved by optimum conditions, and these conditions were used for the determination of NP-G in human urine samples. Moreover, because the de-conjugation process by enzyme and extraction process by SBSE method were simultaneously performed, it succeeded in the development of sample preparation for high throughput.

3.3. Figures of merit of SBSE with in situ de-conjugation and TD-GC-MS

The retention time of NP was from 11.2 to 11.6 min. Because NP presents isomers in technical mixtures, some peaks were observed in the SIM chromatogram (*m/z* 135). The quantification of NP was performed using the sum of all peaks area. The calculated limit of detection (LOD) of NP in the human urine sample by SBSE with in situ de-conjugation and TD-GC-MS was 0.2 ng ml^{-1} (1.0 nM) with the ratio of the compound's signal to the background signal (*S*/*N*) of 3. In addition, the limit of quantification (LOQ) when *S*/*N* > 10 was 1.1 ng ml^{-1} (5.0 nM) for NP. The peak area ratios with respect to the internal standard were plotted, and the response was found to be linear over the calibration range between 5 and 500 nM with a correlation coefficient (*r*) of 0.999. Moreover, we compared SBSE with in situ de-conjugation and SBSE without de-conjugation that was used in our previous study [35], and found that the sensitivity was almost the same. However, the present method succeeded in shortening the operation time significantly. Therefore, SBSE with in situ de-conjugation was superior to SBSE without de-conjugation.

The recovery and precision of the present method were assessed by replicate analysis ($n = 6$) of human urine samples spiked at 20 and 100 nM levels of NP-G, with correction using the added internal standard. The non-spiked and spiked samples were analyzed by SBSE with in situ de-conjugation and TD-GC-MS. The recovery was calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using the calibration curve obtained from standard solutions with the internal standard. The average recoveries in the human urine samples spiked with NP-G at levels of 20 and 100 nM were 104.1 (R.S.D. 7.1%) and 100.6% (R.S.D. 9.2%), respectively. Therefore, the present method may be applied to the precise determination of trace amounts of NP-G in human urine samples.

3.4. Application

We measured the concentrations of NP-G in six urine samples (A–F) obtained from healthy volunteers. NP could not be detected in all the urine samples by SBSE without β-glucuronidase de-conjugation. By contrast, the concentration of NP was 1.5 (A), 1.1 (B), 1.8 (C), 1.8 (D), 2.1 (E) and <1.1 ng ml^{-1} (F) by SBSE with in situ de-conjugation. The typical chromatograms of a human urine sample (volunteer E and F) are shown in Fig. 4. The results suggested that some human was exposed to NP. Taken together, the present method is simple, accurate and highly sensitive for detecting NP technical isomers in human urine samples. Moreover, the measurement of NP-G in human urine sample with the present method has potential for use in the evaluation of the level of exposure to NP.

4. Conclusions

The determination of trace amounts of NP-G in human urine samples using SBSE with in situ de-conjugation and TD-GC-MS was investigated. The present method enabled de-conjugation during SBSE, thereby shortening the operation time. Using the present method, the calculated LOD (*S*/*N* = 3) and LOQ (*S*/*N* > 10) for NP in human urine samples were 0.2 ng ml^{-1} (1.0 nM) and 1.1 ng ml^{-1} (5.0 nM), respectively. The average recoveries were104.1 (R.S.D. 7.1%) and 100.6% (R.S.D. 9.2%) in the urine samples spiked with NP-G at levels of 20 and 100 nM, respectively, with correction using the added isotopically labeled internal standard. This simple, accurate and highly sensitive method is expected to have potential applications in various biological samples. In addition, it is expected that other conjugate compounds can also be applied by this SBSE with in situ de-conjugation method.

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