Deconjugation Characteristics of Ethylparaben Conjugates in Human Urine by Indirect Liquid Chromatography Tandem-mass Spectrometry

Yoshiyuki HARADA^{1,2)}, Shigeru SUZUKI¹⁾ and Kazuko KODAMA¹⁾

¹⁾Graduate School of Bioscience and Biotechnology, Chubu University
(1200 Matsumoto, Kasugai, Aichi 487-8501 Japan)

²⁾General Tokai Technology Center
(2-710 Inokoishi, Meito, Nagoya, Aichi 465-0021 Japan)

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Summary

An indirect quantitation method based on liquid chromatography tandem-mass spectrometry (LC/MS/MS) has been developed for the analysis of glucuronic acid and sulfuric acid conjugates of ethylparaben, for which standard reference reagents are not available. This method was then used for determining both the ethylparaben conjugates in human urine. The decay of these two conjugates in urine samples containing β -glucuronidase/arylsulfatase appeared to follow the first-order-like reaction kinetics; 4-hydroxybenzoic acid was not produced in the reaction. In addition, a multiple regression (MR) equation was obtained ($R^2_{adj} = 0.99$, $P < 0.01$) for the increase in ethylparaben concentration and decrease in the peak areas of its glucuronic acid and sulfuric acid conjugates. In the case of methylparaben and its glucuronic acid and sulfuric acid conjugates, a higher P -value was obtained in the MR equation for the glucuronic acid conjugate, owing to the low urinary concentration of methylparaben. Furthermore, this method was applied to determine the concentration of ethylparaben and its two conjugates in urine samples. Following the ingestion of an ethylparaben-containing supplement drink, ~16% of ethylparaben was excreted as a mixture of ethylparaben and its glucuronic acid and sulfuric acid conjugates in the first morning urine of the subject on the following day.

Key words: Paraben, human urine, conjugate, indirect quantitation, liquid chromatography tandem-mass spectrometry

INTRODUCTION

Para-hydroxybenzoic acid esters (parabens) are widely used as antimicrobial agents and preservatives in cosmetics, pharmaceutical products, and foods; in Japan, approximately 4,000 tons of parabens were imported and produced in 2013 and 2014¹⁾. The concentrations of parabens in foods are controlled by the Food Sanitation Act²⁾, which regulates the contents of ethyl-, *iso*-propyl-, *n*-propyl-, *iso*-butyl-, and *n*-butyl-parabens as food additives. For such regulations, the concentrations of these species are defined as equivalents of *para*-hydroxybenzoic acid, with examples including 0.25 and 0.10 g/L in soy sauce and vinegar, and 0.1, 0.012, and 0.20 g/kg in non-alcoholic beverages and syrup, rind of fruit or fruit vegetable, and fruit sauce.²⁻⁴⁾ Parabens exhibit a broad spectrum of activity against molds, fungi, and bacteria, and show most potent activity against Gram-negative bacteria. Their antimicrobial properties increase in strength with increasing alkyl chain length. Parabens also exhibit estrogenic effects mediated by their phenolic hydroxyl groups, which bind in a similar manner to those of meso-hexaestrol⁵⁾. Indeed, Nishihara et al.⁶⁾ reported that parabens bind to estrogen receptors *in vitro*, while Oishi⁷⁾ reported an *in vivo* decrease in sperm counts and testosterone levels

in male rats exposed to parabens during fetal development.

However, paraben exposure routes and levels differ between humans and experimental animals. Upon exposure to parabens through dermal or oral uptake, parabens can be released from the human body into the environment, followed by metabolism within the organism's cells, excretion into the urine, and subsequent disposal through the sewage system. To date, the levels of parabens and their metabolites have been measured in the environment⁸⁻¹¹⁾, wastewater systems^{9, 16)}, human cells, and urine samples¹²⁻¹⁵⁾. More specifically, the methyl- and propyl-paraben levels in environmental water were 262 and 25 ng/L in Aichi Japan⁹⁾ and 466 and 144 ng/L in Poland¹⁰⁾, respectively, while higher concentrations were detected in a river in Brazil, with methyl-, ethyl-, propyl-, and butyl-parabens being detected at levels of 8.0, 5.8, 13.1, and 15.1 μ g/L, respectively¹¹⁾.

Urinary paraben levels have also been measured as intact species^{12, 13)}, hydrolysis products^{14, 16)}, and conjugates of glucuronic and sulfuric acids^{14, 15)}. To identify such paraben conjugates, a method was previously reported where a precursor ion scan method was coupled with accurate mass analysis¹⁷⁾; it was found that ethylparaben taken up into the human body was excreted in the urine as a mixture of the paraben itself, its hydrolyzed derivatives, and its conjugates of gluc-

uronic and sulfuric acids, in addition to other unidentified species. Although it appeared that the majority of ethylparaben was conjugated with both glucuronic and sulfuric acid in urine, these conjugates could not be directly determined, as they are difficult to synthesize and are not readily commercially available, and therefore, no appropriate technique could be developed using such standards.

Thus, we herein present the development of an indirect quantitation method for the glucuronic acid and sulfuric acid conjugates of parabens based on liquid chromatography tandem-mass spectrometry (LC/MS/MS) and its subsequent application to the mass balance analysis of urinary metabolites of ethylparaben following the consumption of an ethylparaben-containing supplement drink.

METHODS

Materials and reagents

Mini-UniPrep filter vials were purchased from General Electric Company (Fairfield, CT, USA). ABS Elut-NEXUS cartridges (200 mg, 6 mL) were obtained from Agilent Technologies (Santa Clara, CA, USA) and were washed sequentially with acetone, acetonitrile, and methanol (6 mL each), and then with distilled water (12 mL), prior to their use as clean-up cartridges. All 4-hydroxybenzoic acid methyl and ethyl esters (both from Biochemical Research) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Methanol (for LC/MS) and acetone (for high-performance liquid chromatography, HPLC) were obtained from Kanto Chemical (Tokyo, Japan). Acetonitrile (for LC), distilled water (for LC/MS), β -glucuronidase/arylsulfatase (Cat. No. 10127060001, from *Helix pomatia* β -D-glucuronide glucuronosohydrolase/aryl-sulfate sulfohydrolase EC 3.2.1.31/EC 3.1.6.1), and methyl-tri-*O*-acetyl-1-*O*-(trichloroacetimidoyl)- α -D-glucuronate (CAS92420-89-8, for Biochemical Research) were purchased from Merck-Millipore (Darmstadt, Germany), Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St Louis, MO, USA), and Junsei Chemical (Tokyo, Japan), respectively.

Analysis

The instrumental conditions employed for LC/MS/MS analysis are summarized in Table 1. A Waters Xevo TQ-S micro-triple quadrupole mass spectrometer coupled with a Waters ACQUITY ultra-performance liquid chromatography (UPLC) H-Class system (Milford MA, USA) was used for the identification and quantification of the target analytes. Chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (I.D. 3.0 mm $\times$ 100 mm, 1.8 μ m, Waters). An aliquot of each extract (5 μ L) was injected into the instrument, and negative ionization mode using selected reaction monitoring (SRM) was employed for analysis of the target analytes.

Multiple-regression analysis was used to determine the relationships of peak area decreases of paraben-glucuronic acid conjugate and paraben-sulfuric acid conjugate (explanatory variables) with the concentration increases of the corresponding parabens (objective variable). MS-Excel (2010) (Microsoft, Redmond, WA) was used for the calculation to minimize the standardized partial regression coefficient.

Sample collection and preparation

The first morning urine of one volunteer was collected following

Table 1 Instrumental conditions of liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Instruments	Waters ACQUITY UPLC H-class			
	LC conditions			
Column	Waters ACQUITY UPLC HSS T3 3.0 $\times$ 100 mm, 1.8- μ m			
Column Temp.	40°C			
Mobile Phase	A) Water			
	B) CH ₃ CN			
Gradient profile	C) 50 mM CH ₃ COONH ₄			
	Time (min)	A (%)	B (%)	C (%)
	0	85	10	5
	1.2	85	10	5
	20	0	95	5
	24	0	95	5
	26	85	10	5
	36	85	10	5
Flow rate	0.2 mL/min			
Sample volume	5 μ L			
Instruments	Waters Xevo TQ-S micro			
	ESI-negative			
Ionization mode	ESI-negative			
Acquisition mode	SRM			
Capillary voltage	0.5 kV			
Cone Voltage	50 V			
Desolvation Temp.	500°C			
Desolvation Flow	700 L/hr			
Cone Flow	50 L/hr			
Collision Energy MS	3 V			

the consumption of an ethylparaben-containing supplement drink the previous night. This experiment was approved by the ethical committee of the Faculty of Bioscience and Biotechnology, Chubu University. The urine sample was divided into 10 mL aliquots and stored at -20°C prior to sample preparation as outlined in Fig. 1. More specifically, the frozen samples were thawed at $\sim 20^{\circ}\text{C}$, centrifuged at 3000 rpm for 10 min, and the supernatant solution was aspirated, homogenized, and divided into two aliquots of 50 mL. The pH of each aliquot was adjusted using a small volume of 23 M formic acid to either pH 4.3 or 5.6; in both cases, high deconjugation reaction rates were individually obtained for the glucuronide and sulfate conjugates of ethylparaben in preliminary tests. Then, β -glucuronidase/arylsulfatase (40 μ L) was added to each resulting aliquot, which was divided into five portions of 10 mL in test tubes and placed in a water bath at 37°C to promote the gradual hydrolysis of the glucuronic acid and sulfuric acid conjugates of the parabens. An aliquot (1 mL) of each solution was then sampled after 0, 1, 3, 10, 30, 60, 120, and 180 min, and acetonitrile (1 mL) was added to each aliquot prior to stirring for 30 s and subsequent clean-up using an ABS Elut-NEXUS cartridge. More specifically, the aliquot (~ 2 mL) was passed through the cartridge, and the solution eluted from the cartridge using methanol (8 mL) was collected and diluted to 10 mL with methanol. Each sample was subjected to direct analysis of the parabens and indirect analysis of the paraben glucuronic acid and sulfuric acid conjugates by LC/MS/MS.

RESULTS AND DISCUSSION

Ethylparaben recovery rate from urine

The experimental recovery of methylparaben and ethylparaben from urine was carried out using seven 10 mL replicates of urine samples spiked with methylparaben and ethylparaben (10 ng each) in

Fig. 1 Analysis scheme of parabens and their conjugates in human urine

addition to the urine control samples, which were prepared for analysis according to the scheme outlined in Fig. 1. The recovery rate was calculated by subtracting the concentrations of methylparaben and ethylparaben in the control sample from that of the spiked sample; the results are shown in Table 2. As indicated, the average recovery rate of methylparaben was 94% with a relative standard deviation (RSD) of 3.7%, while that of ethylparaben was 114% with an RSD of 4.7%. The limit of detection (LOD) and the limit of quantification (LOQ) of methylparaben were 13 µg/L and 38 µg/L, respectively, and the LOD/LOQ of ethylparaben were, respectively, 22 µg/L and 66 µg/L. With these measures of accuracy and precision of the recovery rates, and the limits of detection (LOD), urinary concentration values

of methylparaben and ethylparaben were used for indirect determination of their glucuronic acid and sulfuric acid conjugates.

Behavior of ethylparaben and its glucuronic acid and sulfuric acid conjugates in urine samples during the simultaneous deconjugation

The concentrations of ethylparaben and its glucuronic acid and sulfuric acid conjugates in the urine samples were measured 1, 3, 10, 30, 60, 120, and 180 min after the deconjugation reaction commenced. Representative SRM chromatograms illustrating the deconjugation of the two ethylparaben conjugates to produce ethylparaben in the urine samples after reaction times of 0 and 180 min are shown in Fig. 2. As

Table 2 Recovery of methylparaben (M.P.) and ethylparaben (E.P.) from urine sample *1 (n = 7)

Substance name	Methyl paraben	Ethyl paraben
Control urine Avg. (µg/L)	7.9	25
Spiked amount [ng]	100	100
Recovered Avg. (µg/L)	102	140
Recovery Avg. [%]	94	115
RSD (%)	3.7	4.7
LOD (µg/L) *2	13	22
LOQ (µg/L) *3	38	66

*1 The recovery experiment was performed by adding methylparaben (M.P.) and ethylparaben (E.P.) to control urine.

*2 LOD=3.29×S.D. (S.D.: standard deviation obtained with the present study)

*3 LOQ=3.04×LOD

indicated, the production of 4-hydroxybenzoic acid was not observed during the deconjugation reaction. In addition, Fig. 3 shows plots of the peak areas corresponding to the two ethylparaben conjugates and the ethylparaben concentrations against time for the deconjugation reaction at pH 4.3 and pH 5.6. Indeed, clear exponential decays were observed between reaction times of 10–180 min. The lower decay rates of the conjugates within the initial 10 min of the reaction were likely due to the delay in reaching the designated reaction temperature. However, the decay rate did not influence the relationship between the increase in ethylparaben concentration and the decrease in peak areas of the glucuronic acid and sulfuric acid conjugates, and the lower decay rates were helpful in accurately measuring the changes in concentration and peak areas over the initial 10 min, in which five replicates of the urine samples were measured after 0, 3, and 10 min. Interestingly, the decay rates of the sulfuric acid conjugate were

E : Ethylparaben G : Glucuronic acid conjugate of ethylparaben
H : 4-hydroxybenzoic acid S : Sulfuric acid conjugate of ethylparaben

Fig. 2 SRM chromatograms illustrating before and after the deconjugation of the two ethylparaben conjugates to produce the intact ethylparaben in urine samples

The deconjugation reaction was performed at pH 5.6

higher at pH 5.6 than at pH 4.3, while those of the glucuronic acid conjugate were comparable under both pH conditions. In addition, the exponential decays observed for the two ethylparaben conjugates suggest that both conjugates decayed according to first-order-like reactions, and that the rate of glucuronic acid conjugate decay was higher than that of the sulfuric acid conjugate. A linear increase in the logarithmic concentration, i.e., an exponential increase in the concentration, of ethylparaben was also observed, as expected from the exponential decay of the ethylparaben conjugates.

The observed results therefore suggest that the glucuronic acid and sulfuric acid conjugates of ethylparaben in the urine samples were successfully deconjugated in the presence of β -glucuronidase/arylsulfatase to produce ethylparaben via two simultaneous first-order-like reactions, with no 4-hydroxybenzoic acid production being observed in either case. Although the deconjugation reaction was performed at pH 4.3 and pH 5.6, the deconjugation reaction rates of the sulfuric acid conjugates were very low at pH 4.3, while at pH 5.6, the deconjugation reaction rates were good enough for both the ethylparaben conjugates. Therefore, all further deconjugation experiments using indirect LC/MS/MS were performed at pH 5.6.

Fig. 3 Peak areas corresponding to the two ethylparaben conjugates and concentrations of intact ethylparaben plotted against the simultaneous deconjugation reaction time

The deconjugation reaction was performed at pH 5.6

Indirect LC/MS/MS method for determination of the glucuronic acid and sulfuric acid conjugates of ethylparaben

Based on the assumption that the decay of the glucuronic acid and sulfuric acid conjugates of ethylparaben took place through simultaneous first-order reactions, multiple regression analysis (MRA) was performed (see Table 3) to estimate the increase in ethylparaben concentration using decreases in the conjugate peak areas as explanatory variables. The multiple regression equation (1) shown in Table 3 was obtained ($R^2_{adj} = 0.99$, $P < 0.01$) for the increase in ethylparaben concentration (C_{ep}) and the decrease in its conjugate peak areas (i.e., glucuronic acid (A_{e-g}) and sulfuric acid (A_{e-s})). However, in general, the coefficients of A_{e-g} , A_{e-s} , and the constant of 5.9 in equation (1) are dependent on analytical conditions and urine samples. Thus, equation (1) is generally written as follows:

$$C_{ep} = A * A_{e-g} + B * A_{e-s} + C \quad (A, B, \text{ and } C \text{ are constant}) \quad (1')$$

Coefficients A and B, and constant C are generally determined with a minimum of three values of C_{ep} , A_{e-g} , and A_{e-s} from SRM measurements. Then, using equation (1)', for which the coefficients and constant A, B, and C have been determined, the concentrations of the glucuronic acid and sulfuric acid conjugates of ethyl paraben can be indirectly determined.

Similarly, the urinary concentrations of the glucuronic acid and sulfuric acid conjugates of methylparaben can also be indirectly estimated by solving simultaneous equations between the decrease in peak areas of the two conjugates and the increase in the quantified concentration of methylparaben (see Table 3). In this case, the multiple regression equation (2) (shown in Table 3) was obtained for the relationship between the methylparaben concentration (C_{mp}) and the peak areas of the glucuronic acid conjugate (A_{m-g}) and the sulfuric acid conjugate (A_{m-s}) ($R^2_{adj} = 0.96$, $P < 0.01$ for sulfuric acid, $P = 0.29$ for glucuronic acid). The higher P -value for the glucuronic acid conjugate was caused by the small peak areas observed, because methylparaben was not intentionally ingested in the experiment, i.e., the higher P -value for the glucuronic acid conjugate of methyl paraben was due to the low concentration and the low sensitivity.

Fig. 4 shows a scatter plot of the ethylparaben equivalent concentration decreases calculated from the peak area decreases of the glucuronic and sulfuric conjugates of ethylparaben with equation (1) and the increases in ethylparaben concentrations. Similarly, a scatter plot of the methylparaben equivalent concentration decreases calculated with equation (2) and the increases in methylparaben concentrations are shown in Fig. 5. Excellent correlations were obtained between the paraben equivalent concentration decreases and paraben concentration increases for both ethylparaben ($R^2_{adj} = 0.99$, abundance ratio = 0.99) and methylparaben ($R^2_{adj} = 0.96$, abundance ratio = 0.96). Therefore, it could be concluded that the concentrations of the methyl- and ethylparaben conjugates can be indirectly quantitated by the method presented herein.

Finally, the concentrations of the glucuronic and sulfuric acid conjugates of ethylparaben present in the first morning urine of one volunteer were measured using the indirect quantitation method following the ingestion of an ethylparaben-containing supplement drink. All measurements were repeated five times and an average concentration equivalent to ethylparaben was taken to obtain ethylparaben, glucuronic acid conjugate, and sulfuric conjugate of 140 ng/mL (RSD 3.3%), 950 ng/mL (RSD 3.4%), and 620 ng/mL (RSD 1.2%), respective-

Table 3 Results of MRA between concentration increases of methyl and ethyl parabens and peak area decreases of those conjugates of glucuronic acid and sulfuric acid (N = 35)

multiple regression equations	$C_{ep}^{*1} = 0.0041A_{e-g}^{*2} + 0.000013A_{e-s}^{*3} + 5.9$					(1)
	$C_{mp}^{*4} = 0.0019A_{m-g}^{*5} + 0.000028A_{m-s}^{*6} + 0.019$					(2)
objective variable	explanatory variables	partial regression coefficient	standardized partial regression coefficient	T- value	p- value	R ² adjusted
C _{ep}	A _{e-g}	0.0041	0.67	6.9	0.000000089	0.9869
	A _{e-s}	0.000013	0.33	3.4	0.0021	
	constants	5.9	-	3.5	0.0013	
C _{mp}	A _{m-g}	0.0019	0.25	1.1	0.29	0.9553
	A _{m-s}	0.000028	0.73	3.2	0.0034	
	constants	0.019	-	0.097	0.92	

The deconjugation reaction was performed under pH5.6.

*1: increase in ethylparaben concentration.

*2 and *3: decrease in the ethylparaben conjugate peak areas of glucuronic acid and sulfuric acid, respectively.

*4: increase in methylparaben concentration.

*5 and *6: decrease in the methylparaben conjugate peak areas of glucuronic acid and sulfuric acid, respectively.

Fig. 4 Scatter plots of calculated and measured values of the ethylparaben concentration increases by deconjugations of its glucuronic acid and sulfuric acid conjugates

The deconjugation reaction was performed at pH 5.6

Fig. 5 Scatter plots of calculated and measured values of the methylparaben concentration increases by deconjugations of its glucuronic acid and sulfuric acid conjugates

The deconjugation reaction was performed at pH 5.6

ly. The total quantity of ethylparaben and its two conjugates excreted in the first morning urine equivalent concentration to ethylparaben was thus 780 μg (RSD 2.3%), which constituted 16% of the ethylparaben ingested from the supplement drink the previous night. However, detailed data relating to the behavior of the ingested parabens were limited, owing to the low urinary concentrations of methylparaben and other parabens, and so it is apparent that additional experiments using more sensitive measurement techniques will be required to interpret the conjugation process and the behavior of the conjugates in greater detail.

CONCLUSION

We herein reported the development of an indirect quantitation method based on LC/MS/MS for determining the concentration of the glucuronic acid and sulfuric acid conjugates of ethylparaben, for which standard reference reagents are not available. The two ethylparaben conjugates were found to undergo deconjugation using β -glucuronidase/arylsulfatase to produce ethylparaben by two simultaneous first-order-like reactions. The production of 4-hydroxybenzoic acid was not observed in either case. In addition, indirect quantitation of the two conjugates was successfully performed by analyzing the relationship between the increase in ethylparaben concentration and the decrease in conjugate peak areas over a range of reaction times ($R^2_{adj}=0.987$, $P<0.01$). Finally, this method was tested using a mixed reagent of β -glucuronidase / allylsulfatase, assuming a small amount of urine sample. This method, which enables calculation of concentrations of two kinds of conjugates by one line of analysis operation, is a method in which the amount of ethylparaben and its glucuronic acid and sulfuric acid conjugate thereof in the urine sample after ingestion of the ethylparaben-containing beverage by human subjects was successfully applied to determine. These results are of particular importance as our developed method can be used in the analysis of parabens and their conjugates in biological samples, to ultimately achieve improved control of the permitted paraben levels in foodstuffs, pharmaceutical products, and cosmetics. The method may contribute to a study such as the Japan Environment and Children's Study, in which exposure measurements of chemicals have been performed with limited sample sizes. However, additional studies are required to elucidate the specific metabolic behavior of such compounds. These studies will be carried out in the near future, and the results will be reported in due course.

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