Direct measurement of the glucuronide conjugate of 1-hydroxypyrene in human urine by using liquid chromatography with tandem mass spectrometry

Direct measurement of the glucuronide conjugate of 1-hydroxypyrene in human urine by using liquid chromatography with tandem mass spectrometry

Kensaku Kakimoto, Akira Toriba*, Takanori Ohno, Mariko Ueno, Takayuki Kameda, Ning Tang, Kazuichi Hayakawa

Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

*To whom correspondence should be addressed.

Tel: +81-76-234-4457, Fax: +81-76-234-4456, E-mail: toriba@p.kanazawa-u.ac.jp

Abstract

To evaluate human exposure to polycyclic aromatic hydrocarbons (PAHs), we developed a rapid, simple and sensitive method for determining 1-hydroxypyrene-glucuronide (1-OHP-G) in human urine. To improve precision, a deuterated glucuronide was used as an internal standard. The method requires only 1 mL of urine. The urine was treated with a mixed-mode anion-exchange and reversed-phase solid-phase extraction cartridge (Oasis MAX). The analytes were analyzed with a C_{18} reversed-phase column with a gradient elution, followed by tandem mass spectrometry with electrospray ionization in negative ion mode. The detection limit of 1-OHP-G (corresponding to a signal-to-noise ratio of 3) was 0.13 fmol/injection. Urinary concentrations of 1-OHP-G determined by this method were strongly correlated (r^2=0.961) with concentrations of 1-hydroxypyrene by conventional HPLC with fluorescence detection.

Keywords: 1-Hydroxypyrene-glucuronide; LC-MS/MS; Urine; Polycyclic aromatic hydrocarbon; Biomarker

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmental carcinogens and/or mutagens [1]. PAHs are formed during the incomplete combustion of organic matter, and humans are exposed to PAHs from various sources, including occupational environments, dietary sources, cigarette smoking, fossil fuels and others [2-4]. PAHs are readily absorbed into the body through the skin, lungs, and gastrointestinal tract. Pyrene is a dominant compound of PAHs [5] and it is readily excreted in urine [6]. Metabolism of pyrene involves the formation of 1-hydroxypyrene (1-OHP) as a phase I metabolite which undergoes phase II metabolism with conjugation to glucuronic acid and sulfate. An *in vitro* study on glucuronidation of 1-OHP under human recombinant UDP-glucuronosyltransferases (UGTs) showed that UGT 1A6, 1A7 and 1A9 are mainly involved in the glucuronidation [7] and the glucuronide levels account for more than 80% of total pyrene metabolites in human urine [8-10]. 1-OHP is a urinary PAH metabolite that has often been used as a biomarker for recent exposure to multiple routes of PAHs. 1-OHP in human urine has been measured with analytical equipments like HPLC with fluorescence detection (HPLC-FL), GC-MS, LC-MS/MS [11-21]. However, these methods need enzymatic hydrolysis of its conjugates to 1-OHP for

generally 2 to 16 hours (overnight) [12, 22]. The hydrolysis step is time-consuming, and its possible incomplete hydrolysis should be taken into consideration [8]. GC-MS methods and some LC-MS/MS methods require a derivatization step in addition to the hydrolysis [15, 17, 20, 21]. Thus, direct measurement of 1-hydroxypyrene-glucuronide (1-OHP-G) would be ideal for the analysis of urine samples. Since 1-OHP-G yields three to five fold higher fluorescence than unconjugated 1-OHP [8, 10], some analytical methods use 1-OHP-G to take advantage of its higher fluorescence. Singh et al. determined urinary 1-OHP-G using reversed-phase SPE as a pretreatment step and HPLC-FL as a quantitative method [8]. Because of its polarity, 1-OHP-G is poorly retained on reversed phase columns and is not completely separated from fluorescent contaminants in urine. Strickland et al. developed an assay using immunoaffinity chromatography as a pretreatment step and synchronous fluorescence spectroscopy as a quantitative method [10]. However, the use of immunoaffinity chromatography may be lacking in flexibility and versatility for general use. Furthermore, the use of an appropriate internal standard is necessary to correct the recovery of 1-OHP-G in pretreatment steps. In this study, a rapid, sensitive and selective LC-MS/MS method using deuterated 1-OHP-glucuronide as an internal standard and an effective pretreatment method for urine samples were developed for qualitative analysis of

urinary 1-OHP-G. The developed method was evaluated by comparison with a conventional HPLC method that uses enzymatic hydrolysis.

2. EXPERIMENTAL

2.1. Materials

1-OHP-G was purchased from the NCI Chemical Carcinogen Repository (MRI, Kansas City, MO, USA). [$^2H_9$]-1-Hydroxypyrene (1-OHP-d_9) was from Chiron (Trondheim, Norway). 1-OHP was obtained from Aldrich (Milwaukee, WI). HPLC grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan) and water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). β-Glucuronidase/aryl sulfatase (type H-2: from *Helix pomatia* β-Glucuronidase activity, 98,000 units/mL and aryl sulfatase activity, 2400 units/mL) was from Sigma (St. Louis, MO, USA). UDP-Glucuronyl transferase 1*1 Human recombinant microsome and 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals and solvents used were of analytical grade.

2.2. 1-OHP-G analysis by LC-MS/MS

The Agilent 1100 series LC system consists of a G1379A degasser, a G1312A binary pump, a G1367A autosampler, a G1316A column oven (all from Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation of 1-OHP-G in urine samples was performed on a ZORBAX Extend-C_{18} column (150 × 2.1 mm i.d., 5 μm, Agilent) with a guard column ZORBAX Extend-C_{18} column (12.5 × 2.1 mm i.d., 5 μm, Agilent). The column temperature was kept at 30°C. A gradient elution using 0.01% NH_4OH in water (eluent A) and 0.01% NH_4OH in methanol (eluent B) was carried out (B, 10% to 90% linear gradient for 20 min) at a flow-rate of 0.2 mL/min. Sample volumes of 5 μL were injected for each analysis.

The mass spectrometric analyses were performed using an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems, CA, USA) equipped with an electrospray ionization (ESI) interface and operated in a negative ion mode. Sensitivity of the selective reaction monitoring (SRM) was optimized by testing with an infusion of 1-OHP-G (1 μM) in a mixture of methanol and water (1/1, v/v) containing 0.01% NH_4OH. The spray voltage was maintained at -4.5 kV. Nitrogen gas was used as the collision gas (CAD) and curtain gas (20 p.s.i.). Zero grade air was used as nebulizer gas (60 p.s.i.) and heater gas (70 p.s.i.). Source temperature was set at 600°C. The mass spectrometer was operated under SRM mode of the transitions at m/z 393.1 → 217.1 for

1-OHP-G and at m/z 402.1 → 226.1 for 1-OHP-d_9-G (internal standard) with dwell times of 1000 ms. The collision energy and declustering potential were set at -38 V and -60 V, respectively. Analyst software (version 1.4, Applied Biosystems) was used to control the LC-MS/MS system, and to acquire and process the data.

2.3. 1-OHP analysis by HPLC with fluorescence detection

The HPLC system for 1-OHP included a DGU-14A degasser a LC-10AD pump, a CTO-10AS column oven, a C-R3A integrator (all from Shimadzu, Kyoto, Japan) and 2475 Multi λ Fluorescence detector (Waters, Milford, MA, USA). According to a previous HPLC method [12], chromatographic separation of 1-OHP and 1-OHP-d_9 (internal standard) in urine samples was performed on a Discovery RP-Amide C_{16} column (250 × 4.6 mm i.d., 5 μm, Supelco, Bellefonte, PA, USA) with a guard column (Discovery RP-Amide C_{16}, 20 × 4.0 mm i.d., 5 μm, Supelco). The elution was run isocratically with a mobile phase consisting of acetonitrile / 10 mM phosphate buffer (pH 7.0) (57/43, v/v) at a flow-rate of 1 mL/min. The column temperature was set at 40°C. The excitation and emission wavelengths were 240 and 387 nm, respectively.

2.4. Preparation of 1-OHP-d_9-G conjugate

1-OHP-d_9-G was synthesized enzymatically from 1-OHP-d_9, using a modification of the method in the previous studies [8, 23]. UDP-Glucuronyl transferase was diluted with 10 mM phosphate buffer (pH 7.4) and was activated with CHAPS on ice for 30 min. A reaction mixture containing 1.5 mM UDP-Glucuronic acid tri sodium salt, 5 mM $MgCl_2 \cdot 6H_2O$, and 500 nM 1-OHP-d_9 and 5 mg/L of the activated enzyme in 20 mL of 50 mM Tris-HCl buffer (pH 7.5) was incubated for 2 h at 37°C. The reaction mixture was loaded onto an OASIS HLB cartridge (Waters) that had been primed with 5 mL of methanol and 10 mL of water. The cartridge was sequentially washed with 10 mL of water. Final elution of the trapped glucuronide was performed with 20 mL of acetonitrile/water (30/70, v/v) and the eluate was then evaporated to dryness.

2.5. Pretreatment of urine samples for LC-MS/MS analysis

The deuterated internal standard (1-OHP-d_9-G) was added to 1 mL of urine and then the urine sample was adjusted to pH 2 with phosphoric acid. The urine sample was then loaded onto an OASIS MAX cartridge (30 mg/1 cc, 30 μm, Waters) that had been

primed with 1 mL of methanol and 1 mL of water. The cartridge was sequentially washed with 1 mL of 50 mM sodium acetate buffer (pH 7) in methanol (95/5, v/v), 1 mL of methanol, 1 mL of methanol/water (80/20, v/v) containing 1.8% HCOOH and 1 mL of acetonitrile/water (30/70, v/v) containing 1.8% HCOOH. The trapped metabolite was eluted with 500 μL of acetonitrile/water (70/30, v/v) containing 3.6% HCOOH, and an aliquot (5 μL) of the eluate was injected into the LC/MS/MS system.

2.6. Pretreatment of urine samples for HPLC with fluorescence detection

Urinary 1-OHP was determined as described previously with a slight modification [12]. A 3 mL aliquot of the sample was adjusted to pH 5.0 with 0.1 M HCl, and then buffered with 6 mL of 0.1 M acetate buffer (pH 5.0). The reaction mixture was mixed with a deuterated internal standard (1-OHP-d_9), incubated for 2 h with β-Glucuronidase/aryl sulfatase (784/19 units) at 37°C, and loaded onto a Sep-Pak C_{18} cartridge (Waters) that had been primed with 5 mL of methanol and 10 mL of water. The cartridge was sequentially washed with 10 mL of methanol/water (40/60, v/v). The trapped metabolite was eluted with 5 mL of methanol and the eluent was evaporated to dryness. The residue was redissolved in 300 μL of acetonitrile/water (1/1, v/v). An

aliquot (10 μL) of the solution was injected into the HPLC system.

2.7. Human studies

Urine samples were obtained from eight smokers (age range 21-23) and thirteen non-smokers (age range 22-34) who lived in Kanazawa City, Japan. The urine samples were collected in the morning and kept at -20 °C until analysis. These urine samples were used for both 1-OHP-G analysis and 1-OHP analysis. The concentration of urinary creatinine was determined with alkaline picrate using a test kit (Wako Pure Chemicals) [24].

RESULTS AND DISCUSSION

3.1. Mass spectra obtained by infusion experiments

The ESI of the 1-OHP-G and 1-OHP-d_9-G were examined in both positive and negative ion modes. Both compounds produced signals only in the negative ion mode, probably because the carboxyl group of gluruconide tends to lose the hydrogen and generate $[M-H]^-$ ions. An enhanced product ion (EPI) scan gave information about the fragmentation pattern of the $[M-H]^-$ ions, and the main product ions of 1-OHP-G and 1-OHP-d_9-G were *m/z* 217.0 and 226.0 $[M-H-176]^-$, respectively (Fig. 1). The fragmentation correspondence between the deuterated and non-deuterated compounds indicated that 1-OHP-d_9-G should be suitable for an internal standard. The product ion at m/z 217.0 represents the loss of the sugar moiety [25]. The ions at *m/z* 175 and 113 in the spectrum correspond to fragment ions of glucuronide [25, 26]. A neutral loss of 176 amu is generally used as a specific method for detecting *O*-glucuronide conjugates in tandem mass spectrometry. Therefore, the $[M-H]^- \rightarrow [M-H-176]^-$ transition was used in the SRM detection of 1-OHP-G and 1-OHP-d_9-G.

3.2. Optimization of mobile phase

In this study, a mixture of water and methanol was used as the mobile phase, and the effect of the addition of HCOOH or NH_4OH on the sensitivity and retention time of the analyte was examined. Addition of 0.1% HCOOH increased retention of the analyte and decreased the detection sensitivity because of the ionization suppression of the glucuronic acid moiety. Addition of 0.01% NH_4OH to the mobile phase had little effect on retention and sensitivity, whereas the addition of NH_4OH decreased the interfering peaks on the chromatogram derived from the urine samples. Therefore, a gradient elution using water and methanol containing 0.01% NH_4OH was applied for the LC/MS/MS analysis. The instrumental detection limit of 1-OHP-G was 0.13 fmol/injection (signal-to-noise ratio >3). This method was found to be more sensitive than previous LC-MS/MS methods for the determination of 1-OHP [16, 19]. In most cases, the fragmentations, loss of CO (28 Da) from the $[M-H]^-$ ion [16, 18] and loss of a water molecule from $[M+H]^+$ [19] of 1-OHP were monitored as the product ion. The product ions used in the previous studies may have been difficult to fragment in the collision cell because of the highly conjugated and rigid chemical structure of 1-OHP [17]. On the other hand, the glucuronide is easy to fragment in the collision cell. Further,

the sensitivity of the present method which does not require a derivatization step was better than or comparable to those of LC-MS/MS methods with derivatization steps [17, 20, 21].

3.3. Development of sample preparation

In our preliminary study of 1-OHP-G, the use of reversed-phase SPE by itself was not enough to retain the analyte and clean up urinary interfering substances because of its high polarity. So we used the OASIS MAX cartridge which contains a mixed mode with reversed-phase and anion-exchange sorbent and which has selectivity for acidic compounds like 1-OHP-G. Standard spiked water or urine samples were adjusted to pH 2 and then applied to the cartridge. Several types of solvents were examined for the elution of the analyte and the removal of interfering substances. 1-OHP-G was not eluted from the cartridge by 50 mM sodium acetate buffer (pH 7)/methanol (95/5, v/v) and methanol. These solvents were useful for the removal of inorganic salts and nonionized hydrophobic contaminants. Further, the cartridge was washed with methanol/water (80/20, v/v) and acetonitrile/water (30/70, v/v) containing 1.8% formic acid which enables the effective elution of anions except for the analyte. Finally,

1-OHP-G was eluted with acetonitrile/water (70/30, v/v) containing 3.6% HCOOH. Figure 2 shows representative SRM chromatograms of the standard solution with the analyte (A, left panel) and the internal standard (A, right panel), and a urine sample of a non-smoker, (B). The physiological components of the urine did not interfere with the identification and quantification of the analytes in the chromatograms.

3.4. Calibration curve and validation

Calibration for standard solutions and standard spiked urine samples was performed in the SRM mode under the optimized conditions described in the experimental section. The calibration curve was linear ($r^2 > 0.999$) when the concentrations of the injected sample were in the range of 0.2 to 100 nmol/L, and the slope was 0.0858 ± 0.0027 (mean ± SD, $n = 3$). The calibration range was based on the urinary concentrations of actual subjects [11, 12, 27]. The matrix effect on the mass spectrometric response was evaluated for the analytes by comparing the slope of the above calibration curve with that of the working curve obtained with the urine matrix. The slope of the curve prepared with four different urine samples was 0.0871 ± 0.0018 (mean ± SD, RSD 2.0%), which was almost identical to that of the curve obtained from the standard

solutions. This clearly showed that the matrix did not affect the calibration curve. Therefore, 1-OHP-G was quantified by using the calibration curve obtained from the standard solution in the following studies. The precision and accuracy of 1-OHP-G determination in human urine with the present LC/MS/MS system were examined by adding two different known amounts of 1-OHP-G to a urine sample. The results are summarized in Table 1. The relative standard deviations (RSD, %) of the intra-day precision study (n = 6) were in the range 3.3 – 6.8, and those of the inter-day assay (n = 4) were in the range of 4.4 – 5.7 for the urine samples spiked at the concentrations of 1000 and 10000 pmol/L 1-OHP-G. The accuracy values (%) of the intra-day study and the inter-day assay were in the range of 98% – 99%. Both intra- and inter-day precision and accuracy were satisfactory for determining 1-OHP-G in human urine.

3.5. Analysis of 1-OHP-G in human urine

The LC/MS/MS method we developed in this study was used to determine the 1-OHP-G concentrations in human urine samples. Figure 2 shows representative SRM chromatograms of a human urine sample. We quantitatively determined 1-OHP-G in human urine samples obtained from 8 smokers and 13 non-smokers (Table 2). The mean

concentrations of 1-OHP-G for smokers and non-smokers were 55.0 ± 21.4 and 27.8 ± 12.3 nmol/mol creatinine (normalized to the concentration of creatinine). The urinary concentrations of 1-OHP-G for smokers were significantly higher than those for non-smokers, and the results obtained by the developed method were comparable to those previously reported [11, 28].

3.6. Correlation between 1-OHP-G by LC-MS/MS and 1-OHP by HPLC-FL

The urine samples were also hydrolyzed in the presence of β-glucuronidase/ aryl sulfatase and then analyzed by conventional HPLC-FL [11]. The total amounts of glucuronide and sulfate conjugates were quantified as free (unconjugated) 1-OHP. A very strong linear correlation (r^2=0.961) was found between the urinary 1-OHP-G and 1-OHP concentrations (Fig. 3). Quantitative data obtained by the HPLC-FL method tend to be higher than those obtained by LC-MS/MS (the slope > 1), presumably due to the presence of 1-OHP sulfate. As 1-OHP-G and free 1-OHP have both been used as biomarkers for PAH exposure in many reports [10, 29-31], this result indicates that glucuronide conjugate account for a large percentage of the total urinary pyrene metabolites, and 1-OHP-G can be used instead of free 1-OHP after deconjugation.

4. CONCLUSIONS

We developed an LC/MS/MS method for the direct, simple and sensitive determination of the concentration of 1-OHP-G in human urine. The method does not require time-consuming hydrolysis and derivatization steps. The method appears to be better than conventional methods based on fluorescence. The separation of 1-OHP-G from fluorescent contaminants in urine is difficult without the use of immunoaffinity or complicated SPE pretreatments. Although deconjugated 1-OHP is well suited for chromatographic separation, the efficiency in the enzymatic hydrolysis step should be taken into consideration. The proposed LC-MS/MS method avoids the problems associated with interfering substances and hydrolysis. Other advantages of the method are that it requires no more than 1 mL of urine, its precision and accuracy can be improved with an internal standard (1-OHP-d_9-G), and the sample treatment time can be shortened by using commercially available 96-well format devices (SPE and autosampler).

Acknowledgements

This study was partly supported by the Industrial Technology Research Grant

Program in 2005 from New Energy and Industrial Technology Development Organization (NEDO) of Japan (ID: 05A21705a), by a Grant-in-Aid for Cancer Research (16-7) from the Ministry of Health, Labour and Welfare, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] IARC. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. IARC, Lyon, 1983.

[2] Y. Liu, L. Zhu, X. Shen, Environ. Sci. Technol. 35 (2001) 840.

[3] D. Hoffmann, I. Hoffmann, J. Toxicol. Environ. Health 50 (1997) 307.

[4] M. Lodovici, P. Dolara, C. Casalini, S. Ciappellano, G. Testolin, Food Addit. Contam. 12 (1995) 703.

[5] Z.H. Zhao, W.Y. Quan, D.H. Tian, Sci. Total Environ. 92 (1990) 145.

[6] F.J. Jongeneelen, F.E. van Leeuwen, S. Oosterink, R.B. Anzion, F. van der Loop, R.P. Bos, H.G. van Veen, Br. J. Ind. Med. 47 (1990) 454.

[7] L. Luukkanen, J. Mikkola, T. Forsman, P. Taavitsainen, J. Taskinen, E. Elovaara, Drug Metab. Dispos. 29 (2001) 1096.

[8] R. Singh, M. Tucek, K. Maxa, J. Tenglerova, E.H. Weyand, Carcinogenesis 16 (1995) 2909.

[9] D.H. Kang, N. Rothman, M.C. Poirier, A. Greenberg, C.H. Hsu, B.S. Schwartz, M.E. Baser, J.D. Groopman, A. Weston, P.T. Strickland, Carcinogenesis 16 (1995) 1079.

[10] P.T. Strickland, D. Kang, E.D. Bowman, A. Fitzwilliam, T.E. Downing, N. Rothman, J.D. Groopman, A. Weston, Carcinogenesis 15 (1994) 483.

[11] T. Chetiyanukornkul, A. Toriba, R. Kizu, T. Makino, H. Nakazawa, K. Hayakawa, J. Chromatogr. A 961 (2002) 107.

[12] T. Chetiyanukornkul, A. Toriba, T. Kameda, N. Tang, K. Hayakawa, Anal. Bioanal. Chem. 386 (2006) 712.

[13] F.J. Jongeneelen, R.B. Anzion, P.T. Scheepers, R.P. Bos, P.T. Henderson, E.H. Nijenhuis, S.J. Veenstra, R.M. Brouns, A. Winkes, Ann. Occup. Hyg. 32 (1988) 35.

[14] S.G. Carmella, K. Le, S.S. Hecht, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 1261.

[15] G. Gmeiner, C. Krassnig, E. Schmid, H. Tausch, J. Chromatogr. B Biomed. Sci. Appl. 705 (1998) 132.

[16] X. Xu, J. Zhang, L. Zhang, W. Liu, C.P. Weisel, Rapid commun. Mass Spectrom. 18 (2004) 2299

[17] Y. Li, A. C. Li, H. Shi, S. Zhou, W.Z. Shou, X. Jiang, W. Naidong, J.H. Lauterbach, Rapid commun. Mass Spectrom. 19 (2005) 3331.

[18] D. Pigini, A.M. Cialdella, P. Faranda, G. Tranfo, Rapid commun. Mass Spectrom. 20 (2006) 1013.

[19] R. Fan, Y. Dong, W. Zhang, Y. Wang, Z. Yu, G. Sheng, J. Fu, J. Chromatogr. B 836 (2006) 92.

[20] L. Xu, D.C. Spink, J. Chromatogr. B 855 (2007) 159.

[21] P. Jacob III, M. Wilson, N.L. Benowitz, Anal. Chem. 79 (2007) 587.

[22] M. Bouchard, C. Viau, Biomarkers 4 (1999) 159.

[23] A. Toriba, H. Nakamura, T. Chetiyanukornkul, R. Kizu, T. Makino, H. Nakazawa, T. Yokoi, K. Hayakawa, Anal. Biochem. 312 (2003) 14.

[24] R.W. Bonsnes, H.H. Taussky, J. Biol. Chem. 158 (1945) 581.

[25] Y. Yang, W.J. Griffiths, T. Midtvedt, J. Sjövall, J. Rafter, J-Å. Gustafsson, Chem. Res. Toxicol. 12 (1999) 1182.

[26] A.M.B. Giessing, T. Lund, Rapid Commun. Mass Spectrom. 16 (2002) 1521.

[27] A. Toriba, H. Kitaoka, R. Dills, S. Mizukami, K. Tanabe, N. Takeuchi, M. Ueno, T. Kameda, N. Tang, K. Hayakawa, C. D. Simpson, Chem. Res. Toxicol. 20 (2007) 999.

[28] S. Hecht, Carcinogenesis 23(2002) 907.

[29] C.C. Abnet, R.B. Fagundes, P.T. Strickland, F. Kamangar, M.J. Roth, P.R. Taylor, S.M. Dawsey, Carcinogenesis 28 (2007) 112.

[30] C. Lai, S. Liou, T. Shih, P. Tsai, H. Chen, T.J. Buckley, P.T. Strickland, J.J.K. Jaakkola, Arch. Environ. Health 59 (2004) 61.

[31] M.J. Roth, Y.L. Qiao, N. Rothman, J.A. Tangrea, S.M. Dawsey, G.Q. Wang, S.H.

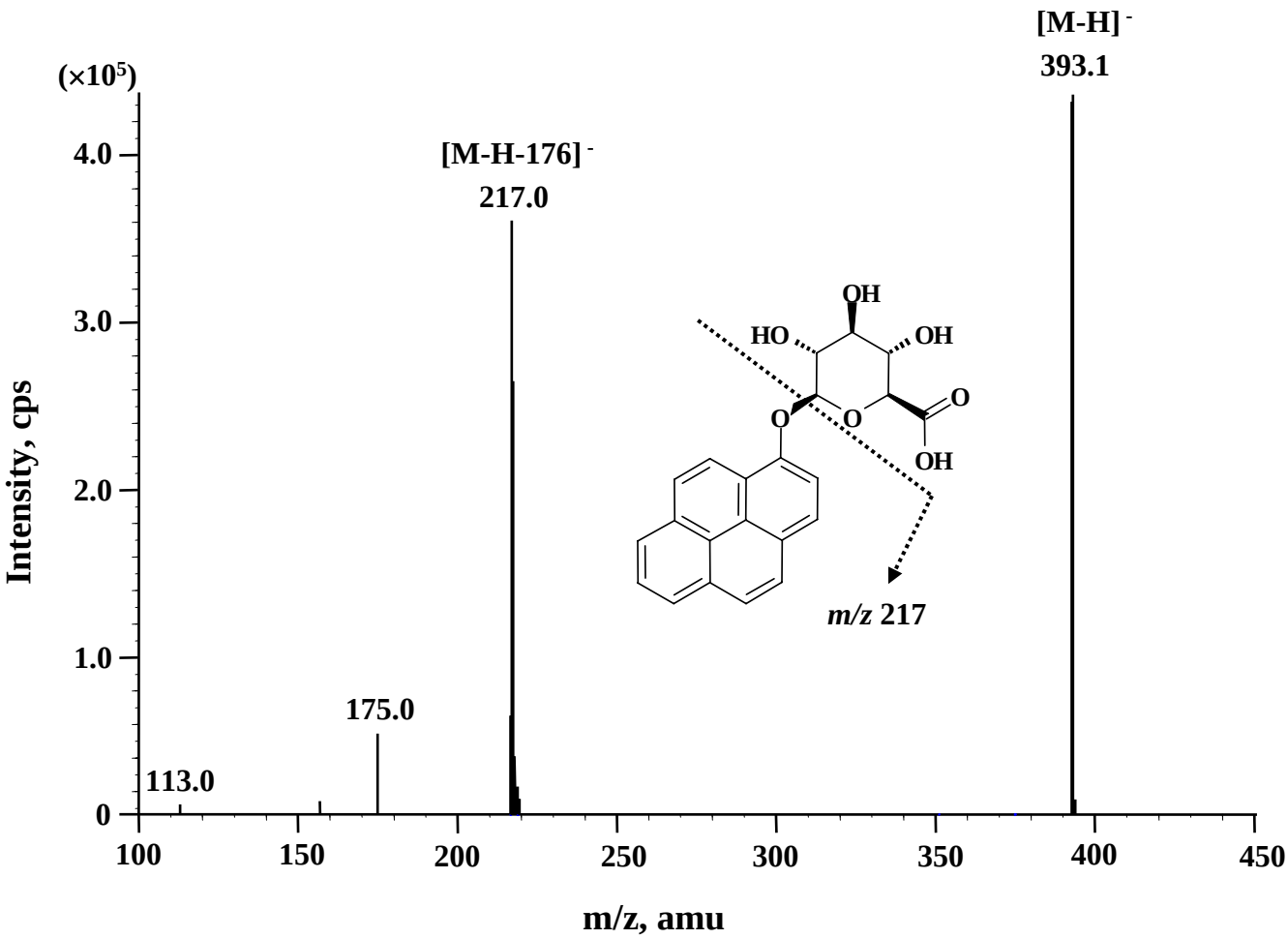
Cho, D. Kang, P.R. Taylor, P.T. Strickland, Biomarkers 6 (2001) 381.

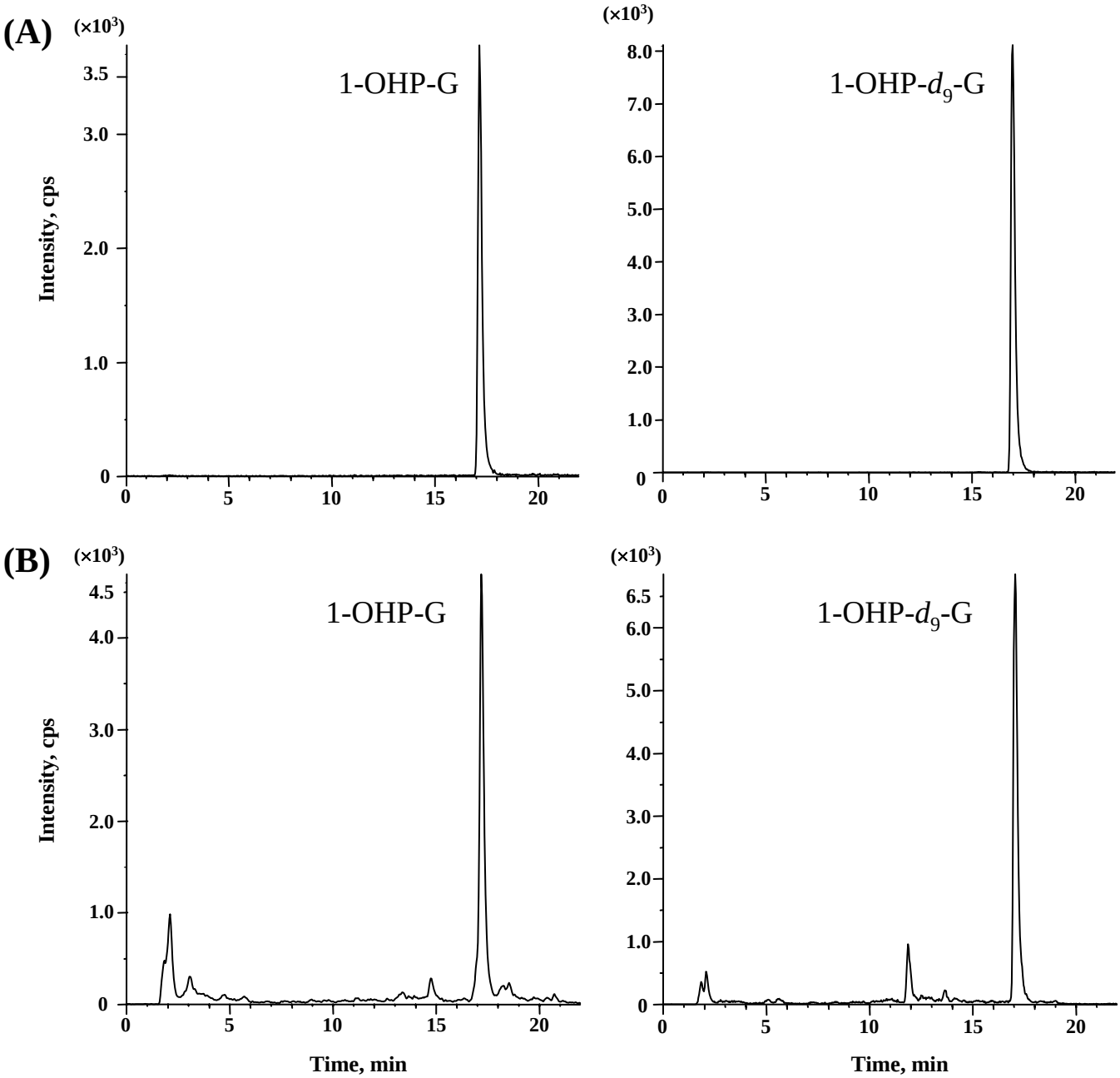
Figure Legends

Fig. 1. MS/MS (EPI) spectra of $[M-H]^-$ ion of standard 1-OHP-G.

Fig. 2. Representative SRM chromatograms (transition *m/z* 393.1→217.1 for 1-OHP-G and *m/z* 402.1→226.1 for the internal standard (1-OHP-d_9-G)) of a standard solution (A) corresponding to 25 fmol 1-OHP-G / injection and a non-smoker urine sample (B) (left panel: 1-OHP-G and right panel: internal standard).

Fig. 3. Correlation between 1-OHP-G and 1-OHP determined by LC-MS/MS and conventional HPLC-FL methods, respectively.


[M-H] -
393.1
(×10⁵)
4.0
3.0
2.0
1.0
0
[M-H-176] -
217.0
175.0
113.0
Intensity, cps
OH
HO
OH
O
O
O
OH
m/z 217
100
150
200
250
300
350
400
450
m/z, amu


(A)
(×10³)
1-OHP-G
1-OHP-d_9-G
Intensity, cps
(B)
Time, min

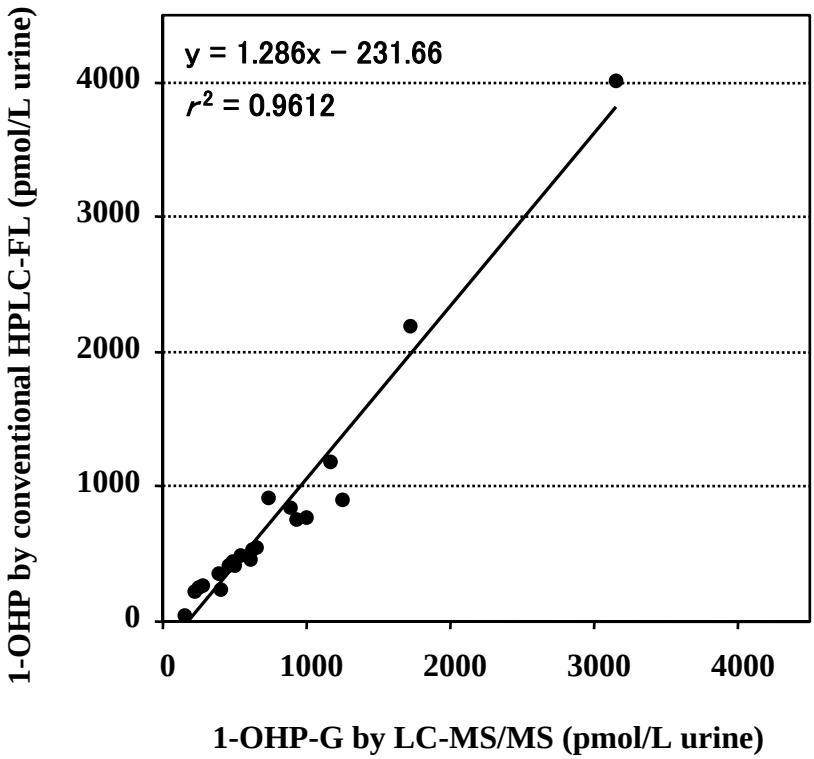

y = 1.286x − 231.66
r2 = 0.9612
4000
3000
2000
1000
0
0
1000
2000
3000
4000
1-OHP by conventional HPLC-FL (pmol/L urine)
1-OHP-G by LC-MS/MS (pmol/L urine)

Table 1. Precision [a] and accuracy [b] in the determination of 1-OHP-G in urine samples

	Intra-day assay (*n*=6)			Inter-day assay (*n*=4)		
Added amounts (pM)	0	1000	10000	0	1000	10000
Found±SD (pM)	632±43	1620±89	10400±340	647±37	1640±86	10500±460
RSD (%)	6.8	5.5	3.3	5.7	5.3	4.4
Accuracy (%)	-	99	98	-	99	99

[a] Precision is expressed as the percentage of relative standard deviation (RSD, %).
[b] Accuracy is expressed as the percentage of accuracy [(mean observed concentration/ spiked concentration) ×100].

Table 2. Urinary concentrations of 1-OHP-G in the study subjects (n = 21)

	Smokers (n=8)	Non-smokers (n=13)
Mean ±SD (nmol/mol of creatinine)	55.0±21.4*	27.8±12.3
Range (nmol/mol of creatinine)	34.6 - 99.7	15.3 - 47.9

* Significantly different from the value of the non-smoker group ($P < 0.01$).