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Toxicology Letters 155 (2005) 403–410
Toxicology Letters
www.elsevier.com/locate/toxlet

Measurement of urinary excretion of 5-hydroxymethyluracil in human by GC/NICI/MS: Correlation with cigarette smoking, urinary TBARS and etheno DNA adduct

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Received 9 September 2004; received in revised form 16 November 2004; accepted 18 November 2004

This article is dedicated to Professor Iwao Ojima for his 60th birthday

Abstract

5-Hydroxymethyluracil (5-HMU) is derived from radiation in addition to endogenous oxidative DNA damage and it is one of the most abundant DNA adducts. Human 5-HMU-DNA-glycosylase has been shown to repair this lesion. Whether urinary levels of 5-HMU is a valid biomarker for oxidative DNA damage in vivo has been investigated. However, controversial results on its relation to cigarette smoking were reported. To facilitate analysis of urinary 5-HMU in epidemiological studies, a highly sensitive and specific assay based on stable isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry was developed. The limit of detection for N^1,N^3-bis(pentafluorobenzyl)-HMU is 10 fg (20 amol) ($S/N = 4$) injected on column and the limit of quantification in urine was 0.7 nM of 5-HMU. Using as little as 10 μL of human urine samples, levels of urinary 5-HMU in 21 healthy volunteers were accurately quantified. No correlation was observed between urinary 5-HMU levels and cigarette smoking. However, there was a statistically significant association between urinary levels of 5-HMU and thiobarbituric acid-reactive substances ($r = 0.71$, $p = 0.0003$). In addition, urinary 5-HMU levels also correlated with urinary levels of 1,N^6-ethenoadenine ($r = 0.54$, $p = 0.01$). These findings suggest that this assay should be valuable in assessing the role of urinary 5-HMU as a biomarker of oxidative DNA damage and repair.
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Keywords: Cigarette smoking; 1,N^6-Ethenoadenine; 5-Hydroxymethyluracil; Mass spectrometry; Oxidative DNA damage; TBARS; Urine

Abbreviations: PFB_2-5-HMU; N^1,N^3-bis(pentafluorobenzyl)-5-HMU; GC/NICI/MS, gas chromatography negative ion chemical ionization mass spectrometry; 5-HMU, 5-hydroxymethyluracil; LC/ESI/MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; PFB, pentafluorobenzyl; SIM, selective ion monitoring; SPE, solid-phase extraction; TBARS, thobarbituric acid-reactive substances

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doi:10.1016/j.toxlet.2004.11.009

1. Introduction

The production of reactive oxygen species within the cells causes damage of biomolecules and it plays an important role in carcinogenesis and aging. An important type of oxidative DNA damage is hydroxylation of DNA bases, leading to formation of 8-oxo-7,8-dihydroguanine; 5-hydroxymethyluracil (5-HMU); 5-hydroxyuracil; 8-oxo-7,8-dihydroadenine, etc. Hydroxylated DNA adducts have been considered as biomarkers of oxidative DNA damage (Shigenaga and Ames, 1991). Cigarette smoking produces various reactive oxygen species and results in elevation of oxidative stress and lung cancer formation (Pryor and Stone, 1993; Hecht, 1999).

Although the biological consequences of 5-HMU in human are not fully understood, specific glycosylase activities have been identified which remove 5-HMU from DNA (Rusmintratip and Sowers, 2000; Baker et al., 2002). Two possible sources account for formation of 5-HMU: via hydroxylation of thymine, forming the HmU:A mismatched base pair, or hydroxylation followed by deamination of 5-methylcytosine, resulting in HmU:G base pair formation. Although the occurrence of the mismatched HmU:A base pair greatly exceeds that of HmU:G, the latter is removed much more efficiently than the former by human 5-HMU glycosylase activities (Rusmintratip and Sowers, 2000; Baker et al., 2002). Thus, the conversion of 5-methylcytosine to 5-HMU is a potential pathway for the generation of 5-methylcytosine to T-transition mutations often found in human tumors.

Increased 5-HMU formation in tissue DNA has been connected with human diseases, and 5-HMU is considered a biomarker of oxidative DNA damage and of breast cancer (Djuric et al., 1991, 1996; Frenkel et al., 1993). Bianchini and co-workers reported that urinary excretion of 5-HMU is higher than that of other oxidized nucleobases, including 8-oxo-7,8-dihydroguanine, and it is significantly higher in smokers than in non-smokers (Bianchini et al., 1998). However, Pourcelot and co-workers showed that urinary excretion of HMU was similar in the smokers and non-smokers groups (Pourcelot et al., 1999). In this study, a highly sensitive and specific assay based on stable isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) was developed to facilitate the analysis of urinary 5-HMU in epidemiological studies and to examine its correlation with the index of lipid peroxidation and lipid peroxidation-induced DNA adducts in the urine.

2. Materials and methods

2.1. Subjects

The subjects were aged between 20 and 68 years. The population consists of 9 smokers and 12 non-smokers, including 3 female non-smokers. The range of number of cigarettes smoked per day was between 5 and 25 and that of years of smoking was between 3 and 50 years.

2.2. Materials

5-Hydroxymethyluracil, pentafluorobenzyl bromide (PFB-Br), diisopropylethylamine, anhydrous methanol, and anhydrous phosphorous pentoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). [$^{13}C_2$,D_2]5-Hydroxymethyluracil (4,5-$^{13}C_2$, 98%; 5,5′-D_2, 98%) was purchased from Cambridge Isotope Laboratories, Andover, MA. Bond Elut C18–OH and Si solid-phase extraction (SPE) columns (500 mg, 3 mL) were from Varian (Harbor City, CA).

2.3. Verification of the structure of PFB_2-5-HMU

To a 4 mL vial containing dried 5-HMU (1.0 mg, 7.8 μmol) was added a solution of diisopropylethylamine (28 μL, 186 mmol) and PFB-Br (20 μL, 132 mmol) in 0.2 mL anhydrous methanol and the reaction mixture was stirred at 42 °C for 1 h. The reaction mixture was evaporated under vacuum and purified by two Si SPE columns to afford 3.0 mg PFB_2-5-HMU (76% yield) as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.30 (C6–H, 1H), 5.28 (N3–CH_2 of PFB, 2H), 5.20 (N1–CH_2 of PFB, 2H), 4.40 (C5–CH_2OH, 2H). NICI/MS (assignment and relative abundance in parentheses) *m*/*z* 321 ([*M*–PFB]$^-$, 100%), 305 ([*M*–PFB-O]$^-$, 25%).

2.4. Assay procedures

Urine samples collected over a 24 h period was stored as 1.0 mL aliquots in 1.5 mL Eppendorff tubes

at the –84 °C freezer. The sample was centrifuged at 23,000 × *g* for 10 min at 4 °C. The creatinine contents were analyzed by a picric acid method (Heinegard and Tiderstrom, 1973). The supernatant (10 μL) of pretreated urine sample was added [$^{13}C_2$,D_2]5-HMU (0.81 ng in 10 μL of water) and 0.1 M potassium phosphate buffer (pH 7.0, 100 μL) and the mixture was loaded on a pre-conditioned C18–OH SPE column. After the sample was loaded, the column was washed with 1 mL of water and collected by elution with the next 2 mL of water. The fraction containing 5-HMU and [$^{13}C_2$,D_2]5-HMU was dried over phosphorous pentoxide, followed by addition of PFB-Br (20 μL) and dii-sopropylethylamine (28 μL) in 0.1 mL anhydrous methanol under argon atmosphere and incubation at 42 °C for 1 h. After the reaction mixture was evaporated to dryness in vacuum, it was dissolved in dichloromethane (0.2 mL) and the pentafluorobenzylated adduct was purified by a Si SPE column pre-conditioned with dichloromethane. The column was washed with 3 mL of 1% methanol in dichloromethane (v/v) and 1 mL of 5% methanol in dichloromethane (v/v) and PFB_2-5-HMU was eluted with 2 mL of 5% methanol in dichloromethane (v/v). The eluant was evaporated, transferred to an insert, evaporated to dryness, reconstituted in 40 μL of acetone and a 1 μL aliquot was analyzed by GC/NICI/MS.

2.5. GC/NICI/MS analysis of PFB_2-5-HMU

The GC/NICI/MS analysis was performed using a Hewlett Packard 6890 GC with 5973 mass selective detector (MSD) with the negative ion chemical ionization (NICI) source (Agilent Technologies, Palo Alto, CA). The filament was operated at 120 eV with the ion source at 150 °C. The analyses were carried out with a cool-on-column inlet, a precolumn (Restek, 2.5 m, 0.53 mm, deactivated silica), and a Rtx-5MS capillary column (Restek, Dellefonte, PA, 0.25 mm × 30 m, 0.25 μm film thickness) inserted into the ion source. Methane (99.999% pure) was the reagent gas with a flow rate of 2.0 mL/min and the pressure at the ion gauge was 2.2×10^{-4} Torr. Helium was used as the carrier gas (99.999% pure) at a flow rate of 1.2 mL/min. The oven temperature of GC was held at 50 °C for the first 2 min, raised to 230 °C at a gradient of 40 °C/min and maintained at 230 °C for 26 min before being raised to 300 °C at a gradient of 40 °C/min and remained at 300 °C for 5 min. PFB_2-5-HMU and [$^{13}C_2$,D_2]PFB_2-5-HMU for were detected and quantified at *m*/*z* 321 and 325, respectively, under the SIM mode for the respective $[M–181]^-$ fragment ions. The quantification of HMU was based on intrapolation of the ratio of the peak area of PFB_2-5-HMU versus [$^{13}C_2$,D_2]PFB_2-5-HMU on the calibration curve.

2.6. Assay calibration

A fixed amount of [$^{13}C_2$,D_2] 5-HMU (0.81 ng) in 10 μL of water was added as internal standard to each sample containing various amounts of 5-HMU ranging from 0, 1, 5, 10, 20, 50, 100, and 300 pg in 0.1 M potassium phosphate buffer (pH 7.0, 100 μL) in a final volume of 120 μL. The samples were processed through the same procedures for urine samples, i.e. adduct enrichment by a C18–OH SPE column, pentafluorobenzylation, Si SPE purification, and GC/NICI/MS analysis. The quantification of 5-HMU was based on intrapolation of the ratio of the peak area of PFB_2-5-HMU versus that of [$^{13}C_2$,D_2]PFB_2-5-HMU to the calibration curve obtained from at least duplicated experiments.

2.7. Measurement of thiobarbituric acid-reactive substances (TBARS)

The level of TBARS in human urine was measured as malondialdehyde (MDA) using a modified method (Liu et al., 1998). Urine was centrifuged at 22,000 × *g* for 30 min. A 280 μL aliquot of the supernatant was mixed with 2 mL of 0.1N HCl, 0.3 mL of 10% phosphotungstic acid, 20 μL of 2 mM butylated hydroxytoluene (BHT), and 1 mL of 0.7% thiobarbituric acid (TBA). The mixture was heated for 60 min at 90 °C, followed by addition of saturated sodium chloride solution (0.5 mL) and extraction of MDA-TBA adduct with 5 mL of 1-butanol twice. The fluorescence intensity of the MDA-TBA complex in the *n*-butanol layer was quickly measured in a fluorescence spectrophotometer using excitation wavelength of 525 nm and emission wavelength of 550 nm. The experiment was performed in triplicates and the urinary concentration of TBARS was expressed as μM MDA. A calibration curve was constructed for each run using 1,1,3,3-tetramethoxypropane as the standard.

2.8. Analysis of urinary 1,N^6-ethenoadenine

Levels of urinary 1,N^6-ethenoadenine were determined by the isotope dilution GC/NICI/MS method published previously (Chen and Chiu, 2003).

2.9. Statistical analysis.

All results are reported as the means ± standard deviation (S.D.). Statistical analysis of two groups was performed by Mann–Whitney *U*-test and $p \leq 0.05$ was considered significant. Spearman rank correlation was used to calculate correlation coefficient. GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA (www.graphpad.com) was used for these analyses.

3. Results

Urinary levels of 5-HMU were analyzed by the isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) after pentafluorobenzylation as N^1,N^3-bis(pentafluorobenzyl)-HMU (PFB_2-5-HMU), which showed good response with GC/NICI/MS despite of its free hydroxyl group. Using modified procedures, the limit of detection (LOD) for standard PFB_2-5-HMU injected on column was improved as 20 amol (or 10 fg with a S/N ratio of 4), compared to the previous report (Kresbach et al., 1989). The entire assay included adduct enrichment by a C18–OH solid-phase extraction (SPE) column, followed by electrophore-labeling and post-derivatization cleanup with a Si SPE column before analysis by GC/NICI/MS. The recovery of the entire assay was 58%. Injecting one fortieths of the sample, the limit of quantification (LOQ) was 1.0 pg (7.1 fmol), defined as the lowest amount of the analyte which showed quantitative linearity in the calibration curve (Fig. 1). The linear correlation coefficient (γ^2) was 0.9995. The *y*-intercept was the peak area ratio of PFB_2-5-HMU versus [$^{13}C_2$,D_2]PFB_2-5-HMU in the control samples. Due to the reported high urinary 5-HMU levels, 10 uL of urine sample was used for each analysis and the adduct levels were all above the LOQ. The concentration quantification limit was thus determined as 0.7 nM.

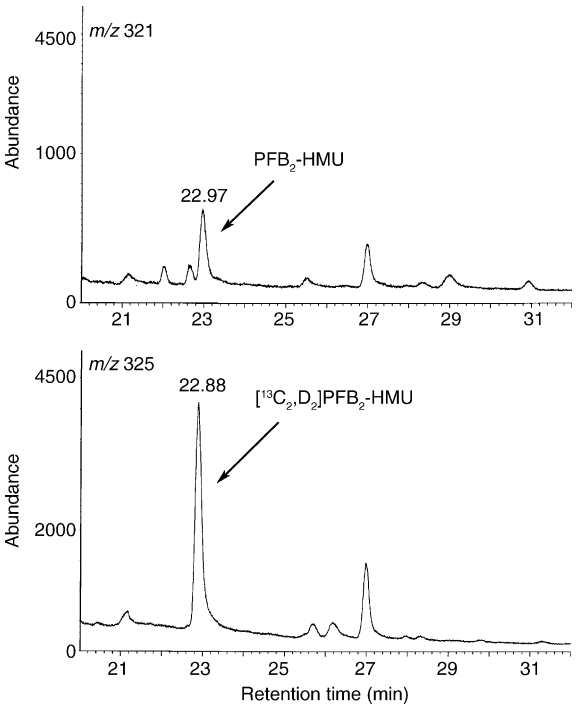

Fig. 1. GC/NICI/MS analysis of PFB_2-5-HMU in a non-smoker's urine. Urine (10 μL) was added [$^{13}C_2$,D_2]5-HMU (0.81 ng), enriched with a C18–OH SPE column, derivatized with PFB-Br, purified by a Si SPE column, and analyzed by GC/NICI/MS under SIM mode as described in the (Section 2). The peak of 22.97 min at *m/z* 321 represents 12 pg (24 fmol) of PFB-5-HMU and the one of 22.88 min at *m/z* 325 is [$^{13}C_2$,D_2]PFB-5-HMU. The result corresponds to a 5-HMU concentration of 13.35 ng/mL.

Fig. 2 showed a representative GC/MS chromatography of PFB_2-5-HMU in a non-smoker's urine sample; that of a smoker's urine was similar (not shown). To separate interference peaks, an isothermal GC condition was used. [$^{13}C_2$,D_2]PFB_2-5-HMU eluted at 22.88 min at *m/z* 325, while PFB_2-5-HMU was 22.97 min at *m/z* 321. The small difference in retention times (ca. 0.11 min) was observed consistently in the calibration curve and in all the urine analyses. Quantification of urinary 5-HMU was based on intrapolation of the peak area ratio of PFB_2-5-HMU versus [$^{13}C_2$,D_2]PFB_2-5-HMU in the calibration curve.

Levels of urinary 5-HMU in the 21 healthy volunteers were in the range between 4.1 ng/mL and 23 ng/mL, which were consistent with the previous reports (Bianchini et al., 1998; Ravanat et al., 1999). Acceptable variances (R.S.D. < 10%) were found for

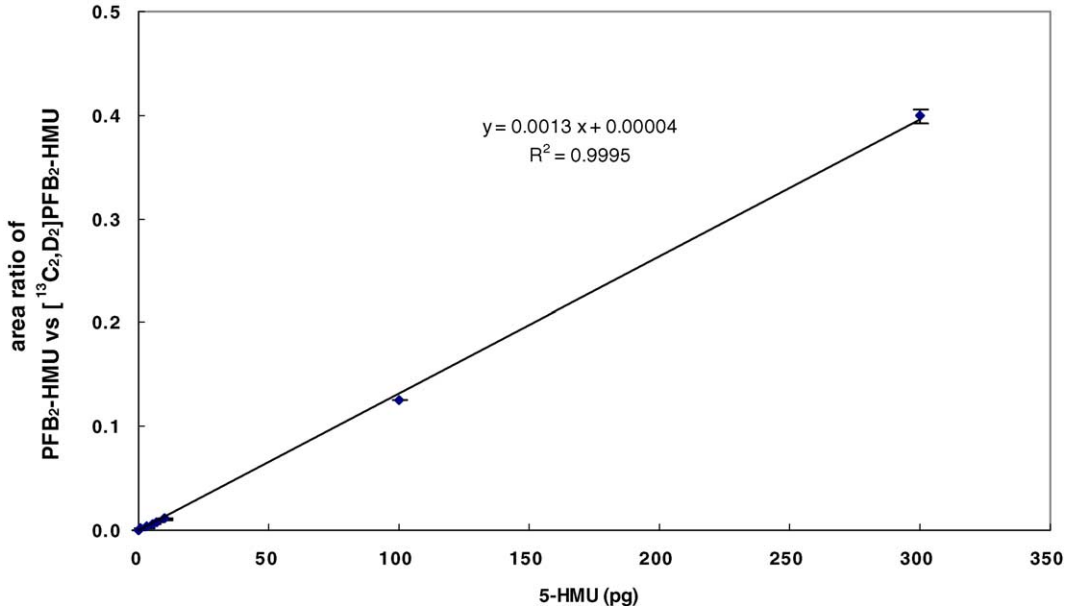

Fig. 2. Calibration curve for the GC/NICI/MS analysis of 5-HMU. Samples containing various amounts (0–300 pg) of 5-HMU were added to a fixed amount of [$^{13}C_2$,D_2]5-HMU (0.81 ng) and subjected to the assay procedures described in the (Section 2) ($r^2 = 0.9995$). The data are combined from at least separate experiments in duplicates. The ratio of each analyte to the internal standard was calculated based on the peak areas.

Table 1
Precision of the GC/MS assay for urinary HMU

Samples	Adduct levels (ng/mL) R.S.D. (%) ($n = 5$)			
	Day 1	Day 2	Day 3	Interday variation R.S.D. (%)
No. 1	18.3 ± 0.3 (1.8)	20.7 ± 0.1 (0.5)	20.3 ± 0.4 (1.9)	5.4
No. 2	8.5 ± 0.9 (10)	7.3 ± 0.1 (1.9)	7.1 ± 0.3 (4.4)	8.0
No. 3	5.7 ± 0.1 (1.6)	6.8 ± 0.3 (4.4)	6.2 ± 0.2 (3.2)	7.4

Values in parenthesis are in percent.

all the samples. The averaged intraday and interday precision of the assay was 3.3% (R.S.D., $n = 5$) and 6.9% (R.S.D., $n = 3$), respectively (Table 1). The concentrations of 5-HMU in smokers' and non-smokers' urine were determined as 95 ± 36 nM and 80 ± 40 nM, respectively. Despite normalization of the adduct levels by creatinine concentration and/or body weight, no statistically significant correlation was found between urinary 5-HMU levels and cigarette smoking using Mann–Whitney *U*-test (Table 2). However, an extremely significant association ($p = 0.0003$) was found between urinary levels of 5-HMU and thiobarbituric acid-reactive substances (TBARS) with a Spearman correlation coefficient (r) of 0.71 (Fig. 3). Furthermore, urinary 5-HMU levels were found to correlate with urinary levels of 1,N^6-ethenoadenine ($r = 0.54$, $p = 0.01$) (Fig. 4), but not 3,N^4-ethenocytosine ($r = 0.34$, $p = 0.13$).

4. Discussion

Compare to ^{32}P-postlabelling technique or immunoaffinity chromatography-based assays for analysis of DNA adducts, mass spectrometry provides chemical characterization of the analyte.

Table 2
Levels of 5-HMU in human urine samples and statistical data

	Smokers[a] ($n = 9$)	Non-smokers[a] ($n = 12$)	p-value[b]
5-HMU (ng/mL)	12.2 ± 4.7 (7.5 – 22.9)	10.3 ± 5.5 (4.1 – 20.1)	0.35
5-HMU (nM)	95 ± 36 (58–178)	80 ± 40 (32 – 156)	0.35
5-HMU/creatinine (nmol/mmol)	10.9 ± 4.7 (7.4 – 23.2)	8.9 ± 2.0 (6.2 – 13.7)	0.32
5-HMU/body weight/ creatinine (pmol/(kg/g)	1.3 ± 0.6 (0.7 – 2.9)	1.2 ± 0.1 (0.6 – 1.9)	0.98

[a] Mean ± S.D. (range).
[b] The p-values were obtained by comparing adduct levels among smokers vs. non-smokers using nonparametric Mann–Whitney U-test.

Incorporation of stable isotope of the analyte, which has identical chemical property as the analyte, provides detailed monitoring of recovery in each step of the analytical procedure and thus ensures accurate quantification of the analyte. Analysis of oxidized bases by GC/MS often used electron impact ionization MS after derivatization with silylating agents. The silylation conditions require high temperature, e g. 110–150 °C, which might cause artifactual formation of oxidized DNA adducts (Halliwell and Dizdaroglu, 1992; Ravanat et al., 1995). In this assay, pentafluorobenzylation was performed at 42 °C and the background levels in the control experiments were very low. The GC/MS assay with electrophore-labeled analyte using negative ion chemical ionization also provides much higher sensitivity than methods using electron impact ionization. Furthermore, the use of disposable SPE columns in this assay eliminated the possibility of carryover from collecting the adduct-containing fractions by HPLC and the tedious cleanup between sample runs (Bianchini et al., 1998; Ravanat et al., 1999). Levels of HMU measured in this study (6.2–23.2 nmol/mmol creatinine or 32–178 nM) are in the same range, 3.2–18.7 nmol/mmol creatinine, as those reported by Bianchini and co-workers (Bianchini et al., 1998) or 121 ± 56 nM by Ravanat and co-workers (Ravanat et al., 1999). The agreement in the range of adduct levels in these three studies suggest that our new assay is reliable and it can be useful in epidemiological studies.

An alternative technique, namely the liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS), has recently been used for analysis of DNA adducts in tissue and biological fluidic samples with high specificity. To the best of

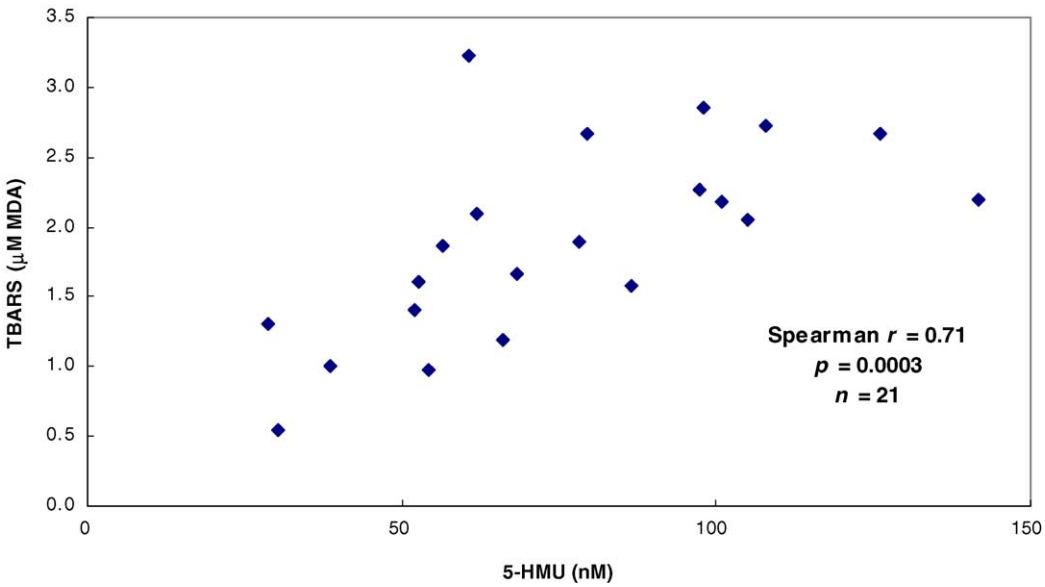

Fig. 3. Correlation between the urinary 5-HMU and TBARS levels of 21 human urine samples.

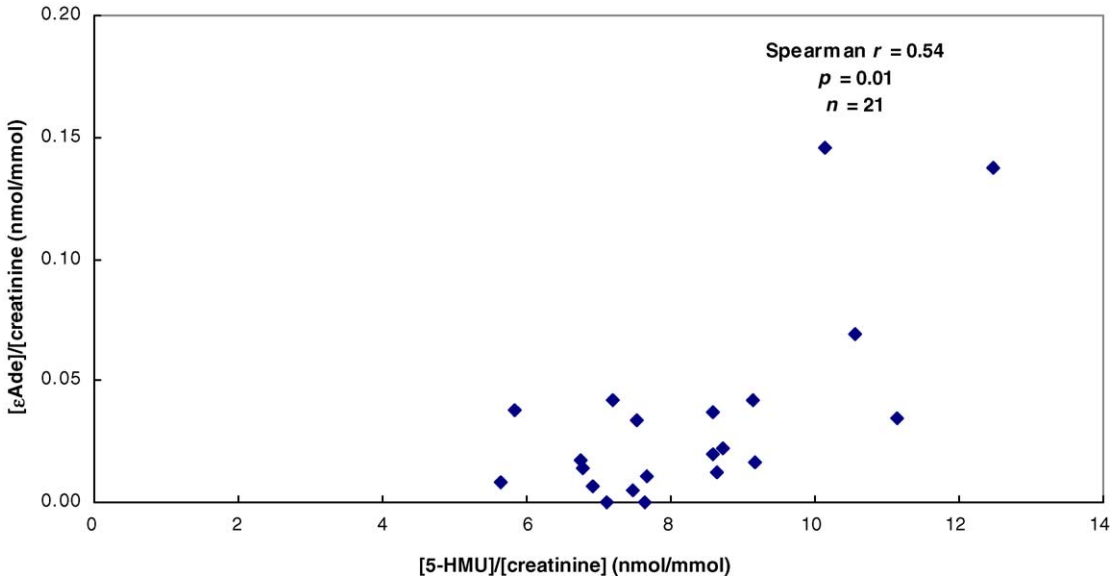

Fig. 4. Correlation between the urinary 5-HMU and 1,N^6-ethenoadenine levels of 21 human urine samples.

our knowledge, there has been no report on analysis of 5-HMU by LC/ESI/MS/MS to date. On the other hand, the deoxyribonucleoside 5-(hydroxymethyl)-2′-deoxyuridine has been measured by LC/ESI/MS/MS in cellular DNA (Frelon et al., 2000). We attempted to develop a LC/ESI/MS/MS assay for urinary 5-HMU, but the sensitivity of this base adduct was poor and severe matrix effect suppressing the analyte signal was observed (Chen et al. unpublished results). Although the GC/MS assay described in this study is somewhat troublesome, requiring adduct enrichment by SPE columns and derivatization steps, it is highly sensitive and quantitative. Nonetheless, the SPE and derivatization steps help in removing the interferences in the complex mixture of urine.

Elevated levels of lipid peroxidation could lead to damage of DNA producing exocyclic etheno DNA adducts, such as 1,N^6-ethenoadenine and 3,N^4-ethenocytosine (Chung et al., 1996). Although our results showed that urinary 5-HMU excretion was not associated with cigarette smoking, it correlated with urinary TBARS, an index of lipid peroxidation, as well as urinary 1,N^6-ethenoadenine, a biomarker of oxidative DNA damage derived from lipid peroxidation (Chen and Chiu, 2003). On the other hand, Faure and co-workers reported that urinary 5-HMU levels were significantly increased by the treatment of adriamycin, an anticancer drug producing reactive oxygen species, with elevated TBARS and lowered antioxidant vitamins in plasma of cancer patients (Faure et al., 1996). Recently, urinary 5-(hydroxymethyl)-2′-deoxyuridine was found to be discriminating of oxidative stress from tobacco smoke (Harman et al., 2003). Although there are other possible sources of urinary DNA adducts, it is accepted that excretion of adducted DNA bases and the deoxyribonucleosides represent the result of repairing DNA damage by base excision repair and nucleoside excision repair mechanisms, respectively. These adducts have been used to estimate DNA adduction in the body, as demonstrated for 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (Loft and Poulsen, 1998; Gackowski et al., 2003). Thus, the urinary 5-HMU assay presented in this study suggests its potential applicability to studies of oxidative DNA damage and repair in humans.

Acknowledgment

This work was supported by grants from National Science Council of Taiwan and from National Chung Cheng University (to H.-J.C.C.).

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