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Uracil in Human DNA from Subjects with Normal and Impaired Folate Status As Determined by High-Performance Liquid Chromatography−Tandem Mass Spectrometry

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A sensitive and selective method for determination of the uracil content in human DNA was first developed on the basis of high-performance liquid chromatography−tandem mass spectrometry. Uracil was excised from DNA using uracil DNA glycosylase. The released uracil was derivatized with 4-bromomethyl-7-methoxycoumarin, thereby forming bis-*N*,*N*′-(4-methylene-7-methoxycoumaryl)-uracil (uracil-MMC). $^{15}N_2$-Uracil was used as an internal standard. The analytes were separated on an Adsorbsphere XL ODS column. A SCIEX API III tandem mass spectrometer equipped with a turbo ion-spray interface was used as the detector. Multiple reaction monitoring using the parent → product ion combinations of *m*/*z* 489 → 232 and 491 → 233 were used to detect uracil-MMC and the internal standard, respectively. The detection limit for this assay is $<1.0 \times 10^{-10}$ mol/L uracil, and the linearity is from 1.0×10^{-10} to 2.5×10^{-6} mol/L. The method was used for determination of uracil in human DNA. Our data show that the uracil levels in human DNA isolated from peripheral white blood cells did not differ between subjects with folate deficiency and subjects with normal red cell folate levels.

Several recent studies[1−3] suggest that low dietary intake and low blood levels of folate are associated with an increased risk of cancer, especially cancer of the colorectum. The mechanism is not fully understood, but one possibility is that folate deficiency leads to increased an uracil content in DNA. This has been explained by impaired conversion of dUMP to dTMP by thymidylate synthase, which in turn leads to incorporation of dUTP instead of dTTP into DNA. Another possibility is that folate deficiency, because of its effect on adenosylmethionine, may lead to increased levels of unmethylated cytosine, resulting in an increased potential for cytosine deamination to uracil.[4] Notably, cytosine deamination may also occur in response to heat or to chemical mutagenesis.[5−7]

Uracil in DNA is excised by uracil DNA glycosylase (UDG) as part of normal DNA repair. The process leaves an apyrimidinic site that is subsequently repaired by sequential action of apurinic/apyrimidinic endonuclease, 5′-phosphodiesterase, DNA polymerase, and DNA ligase. Since the DNA polymerases cannot distinguish between dUTP and dTTP, an increase in the dUTP:dTTP ratio, as observed in folate deficiency, will propagate into uracil misincorporation during both DNA replication and repair synthesis. The action by UDG leaves a transient nick in DNA, but with two closely located opposing nicks, it may lead to DNA strand breaks, chromosome breaks, subsequently increasing the cancer risk in humans.[2,8−10]

There are reports on widely different concentrations of uracil in DNA isolated from both normal and folate-deficient cells. Some,[2,3] but not all,[11] studies show that folate deficiency is associated with a marked increase in uracil content. This apparent discrepancy may partly be explained by the design of the study and the cell types used, but it may also be related to the method employed.

The uracil content in DNA has been assessed using different methods, including single-cell gel electrophoresis,[12] anion-exclusion chromatography,[3] and HPLC with UV detection.[4] Recently, Blount et al. developed a sensitive assay for the uracil content in human DNA using gas chromatography−mass spectrometry.[13] During recent years, LC−MS or MS/MS has proven to be an

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alternative to conventional chromatographic methods.[14−16] In this paper, we present a sensitive and highly selective HPLC−MS/MS method for determination of the uracil content in human DNA.

MATERAILS AND METHODS

Materials. Uracil, 18-crown-6, potassium carbonate, 4-bromomethyl-7-methoxycoumarin (BrMMC), proteinase K, calf thymus DNA, and acetone were purchased from Sigma Chemical Company (St. Louis, MO). Internal standard, $^{15}N_2$-uracil, was obtained from MSD Isotopes (Montreal, Canada). Uracil DNA glycosylase (1unit/μL) was from Epicentre Technologies (Madison, WI). QIAquick PCR purification kit and QIAquick blood kit were purchased from QIAGEN (Hilden, Germany). Water, double distilled and purified on a Milli-Q Plus water purification system (Millipore, Bedford, MA), was used for preparation of all aqueous solutions.

DNA Extraction. The QIAquick blood kit was used for extraction of DNA from human blood samples (2−5 mL) according to the manufacturer's instructions and reference.[17] DNA was dissolved in TE buffer (10 mM Tris/1 mM EDTA, pH 7.5), and its concentration was determined spectrophotometrically assuming that 50 μg/mL of DNA has 1.0 OD at 260 nm.

Preparation of Standard DNA. Synthesis of the standard DNA containing uracil was accomplished by a PCR procedure in which dTTP was replaced by dUTP. A 198 bp DNA fragment from the MTHFR gene was amplified using the primers 5′-GGAGCTTT-GAGGC-TGACCTGAA-3 and 5′-TGAAGGAGAAGGTGTCT-GCGG-GA-3′.[18] The PCR reaction mixture contained 10 mM Tris/HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin; 0.1% Triton X-100; 125 μM of each dNTP (in which dTTP was replaced with dUTP); 0.2 μM of each primer; 2 U Taq polymerase (Super Taq, HT Biotechnology Ltd., U.K.); and ∼1 μg template DNA in a final volume of 100 μL. The PCR product was purified using the QIAquick PCR purification kit according to the manufacturer's instructions. A second PCR was performed using the first PCR product as a template and the same PCR parameters listed above. The PCR product was purified using the QIAquick PCR purification kit. Uracil content in the PCR product was determined by HPLC−MS/MS (see below), and the uracil-to-DNA ratio (uracil-to-DNA base mole ratio) was 0.14, that is, close to the theoretical value of 0.17, according to the DNA base sequence. This PCR product containing uracil was stored at −20 °C. A known amount of this DNA was analyzed as a positive control with every set of samples.

Uracil DNA Glycosylase Digestion. Uracil was excised from the DNA using uracil DNA glycosylase (UDG).[19−20] Human DNA (5−20 μg) was incubated in 50 μL of TE buffer for 60 min at 37 °C in the presence of 0.3 units of UDG. Then, DNA samples were heated at 95 °C for 3 min to break the sugar backbone and inactivate UDG. At this stage, 1 pM of $^{15}N_2$-uracil was added as an internal standard, and the sample was lyophilized for 2 h.

Derivatization. The dried DNA sample was resuspended in 1.2 μL of water to dissolve the uracil. Then, 80 μL of acetone and 40 mg of solid potassium carbonate were added, and the mixture was gently shaken for 3 min. Finally, 50 μL of 1 mg/mL BrMMC and 50 μL of 2.5 mg/mL 18-crown-6 were added. The samples were gently shaken at room temperature (∼22 °C) for 1 h and then centrifuged at 10000 rpm for 1 min. The liquid was transferred to another 1.5-mL Eppendorf tube and lyophilized for 20 minuets. The dried sample was dissolved in 200 μL of 10 mM acetic acid:acetonitrile (1:1) and was stored until analysis.

HPLC−MS/MS. An API 365 triple quadrupole mass spectrometer (PE SCIEX, Thornhill, Ontario, Canada) was coupled to an HPLC system (series 200 micro LC pump, Perkin-Elmer). The turbo ion spray was used as an ion source, and the temperature was set at 450 °C. The mass spectrometer was operated in a positive ion mode, and the voltages were optimized for maximal sensitivity. The parameters were as follows: IS source voltage, 5.0 kV; nebulizer gas setting, 9; curtain gas setting, 8; collision cell gas (nitrogen), 2; focusing ring, 140 V; orifice, 105 V. Multiple reaction monitoring (MRM) mode was used for detection of derivatized uracil with a dwell time of 150 ms. The first quadrupole (Q1) was set to transmit the precursor ions MH^+ at m/z 489 for the derivatized uracil and 491 for the derivatized $^{15}N_2$-uracil, used as an internal standard**.** The product ions were monitored in the third quadrupole (Q3) at m/z 232 and 233 for uracil and $^{15}N_2$-uracil, respectively.

Reversed-phase HPLC was performed on an Adsorbsphere XL ODS column (150 × 4.6 mm, 5 μm, Alltech), and the injection volume was 20 μL. The column was eluted isocratically at a flow rate of 1 mL/min with a mobile phase consisting of 5 mM acetic acid:acetonitrile (3:7). The column was maintained at room temperature (∼22 °C).

RESULTS AND DISCUSSION

Mass Spectra of Uracil and Its Methylenemethoxycoumaryl Derivative. First, we tried to develop a LC−MS/MS assay that could determine underivatized uracil in human DNA. Using turbo ion-spray ionization or atmospheric pressure chemical ionization (APCI), we found that uracil had a measurable response in positive ionization mode (parent → product ion combinations of m/z 113 → 69), but the assay was not sufficiently sensitive to determine the low levels of uracil in human DNA.

BrMMC has been used to derivatize 5-fluorouracil prior to HPLC with fluorescence detection[21−23] or MS/MS.[24] On the basis of the published results,[24] we expected that BrMMC would react with uracil at two locations, forming bis-*N*,*N*′-(4-methylene-7-methoxycoumaryl)-uracil (uracil-MMC) (Figure 1).

Using turbo ion-spray ionization in positive ion mode, uracil-MMC generated a strong signal at m/z 489. Figure 2A displays the full scan mass spectrum obtained after infusion of the uracil-MMC dissolved in 50% acetonitrile in water. The ion at m/z 303 derived from the potassium adduct ion of 18-crown-6. The MS data confirmed the structure of the uracil-MMC displayed in Figure 1.

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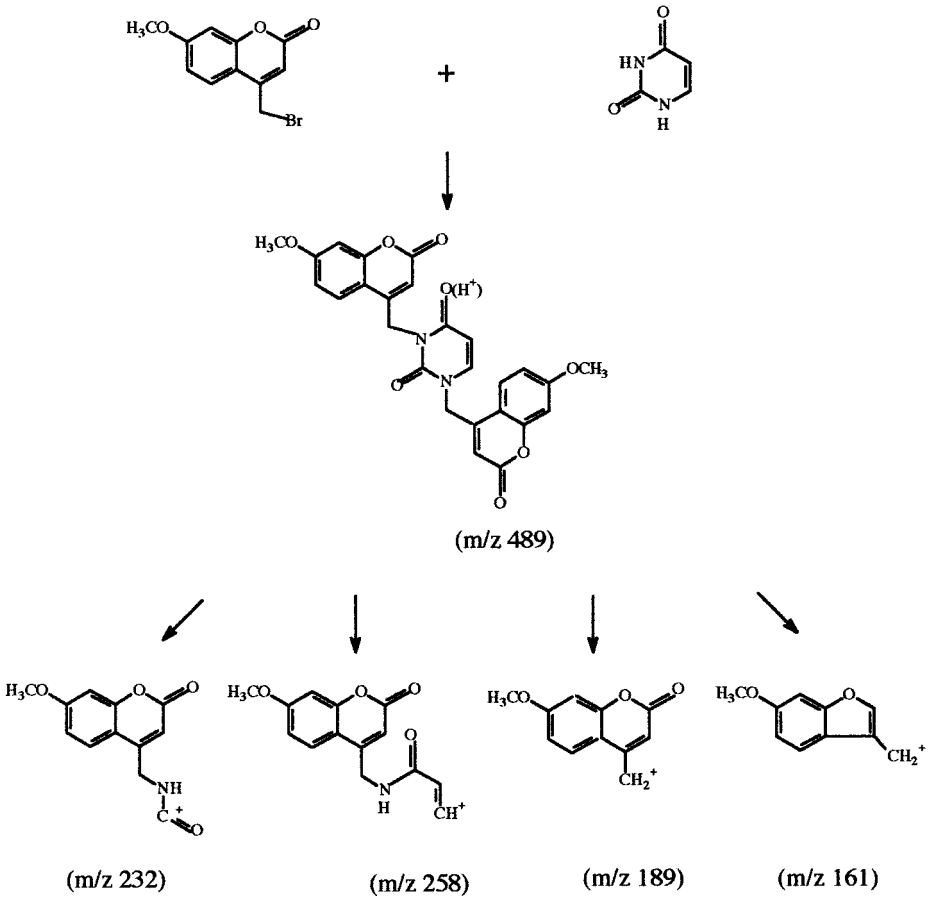

Figure 1. Derivatization of uracil with 4-bromomethyl-7-methoxycoumarin and mass fragmentation of the derivative.

In the multiple reaction monitoring (MRM) mode, the uracil-MMC yielded four product ions, that is, at m/z 161, 189, 232, and 258 (Figure 2B). The ions at m/z 161 and 189 derive from the 4-methyl-7-methoxycoumarin moiety of the molecule (Figure 1) and were similar to those that were reported in earlier studies on derivatized 5-fluorouracil.[24] The two other product ions at m/z 232 and 258 derive from the uracil molecule itself (Figure 1).

The full scan and product ion spectra of the derivatized $^{15}N_2$-uracil, used as an internal standard, were identical to uracil, except that the base peaks were shifted up 1−2 mass units. The parent ion was at m/z 491, and the product ions were at m/z 161, 189, 233, and 259.

HPLC−MS/MS. The uracil-MMC derivative was subjected to chromatography on an Adsorbsphere XL ODS column. Using an isocratic system with 5 mmol/L acetic acid:acetonitrile (3:7) as mobile phase, uracil-MMC (or $^{15}N_2$-uracil-MMC) eluted as a sharp, symmetrical peak with a retention time of 2.26 min. Figure 3 shows the MRM elution profiles of the $^{15}N_2$-uracil-MMC peak at 489 → 232, 491 → 233, 489 → 161 and 489 → 161 m/z. The MRM profiles of uracil-MMC were similar to that of $^{15}N_2$-uracil-MMC (data not shown).

We examined whether the internal standard $^{15}N_2$-uracil-MMC (10^{-8} mol/L) resulted in detectable signals in the same channels as uracil-MMC. A small peak was observed for the parent → product ion combinations m/z 489 → 161 and 489 → 189, that is, the two product ions deriving from the 4-methyl-7-methoxycoumarin part of the molecule. In contrast, the product ions deriving from the uracil molecule (m/z 232 and 258) remained blank, as

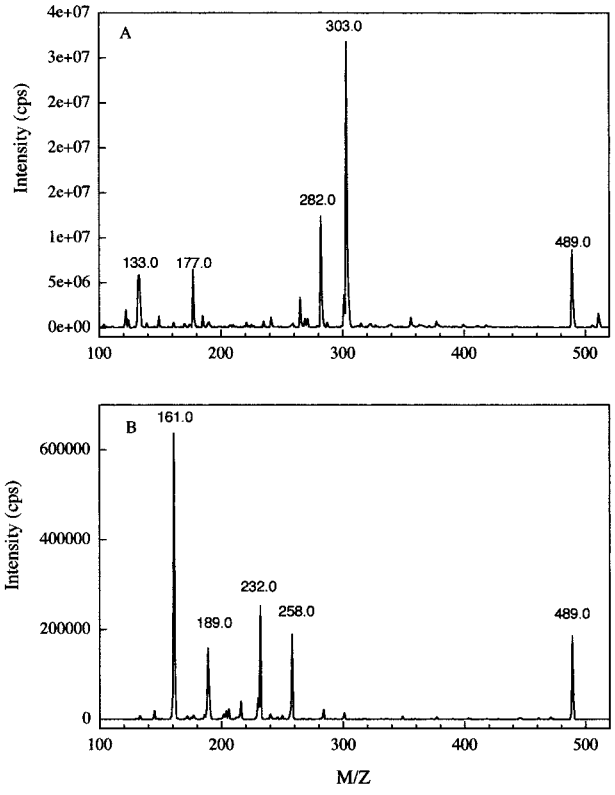

Figure 2. Full scan mass spectrum (upper panel) and product ion mass spectrum of derivatized uracil (lower panel) obtained by infusion of derivatized uracil (10^{-4} mol/L) in 50% acetonitrile.

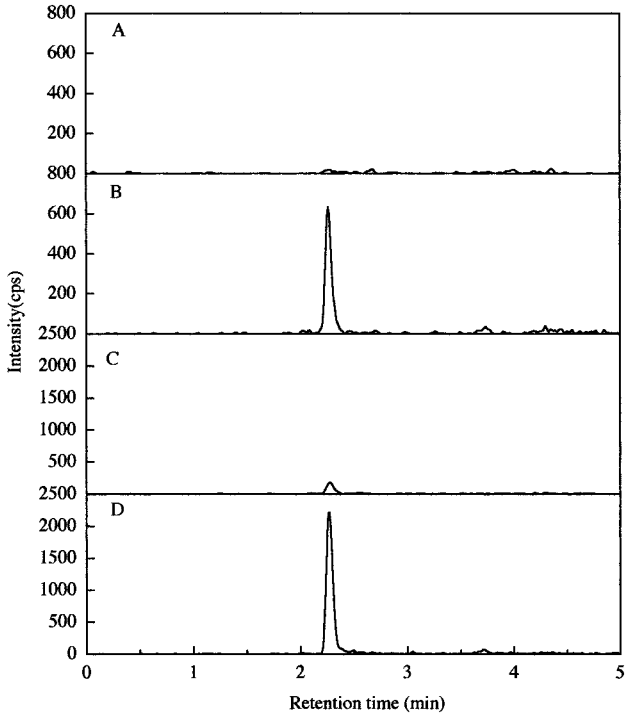

Figure 3. LC−MS/MS chromatograms of $^{15}N_2$-uracil-MMC in the MRM mode. Sample concentration was 1×10^{-8} mol/L, and 20 μL was injected. Panels A−D show the MRM chromatograms at *m*/*z* 489 → 232, 491 → 233, 489 → 161, and 491 → 161, respectively.

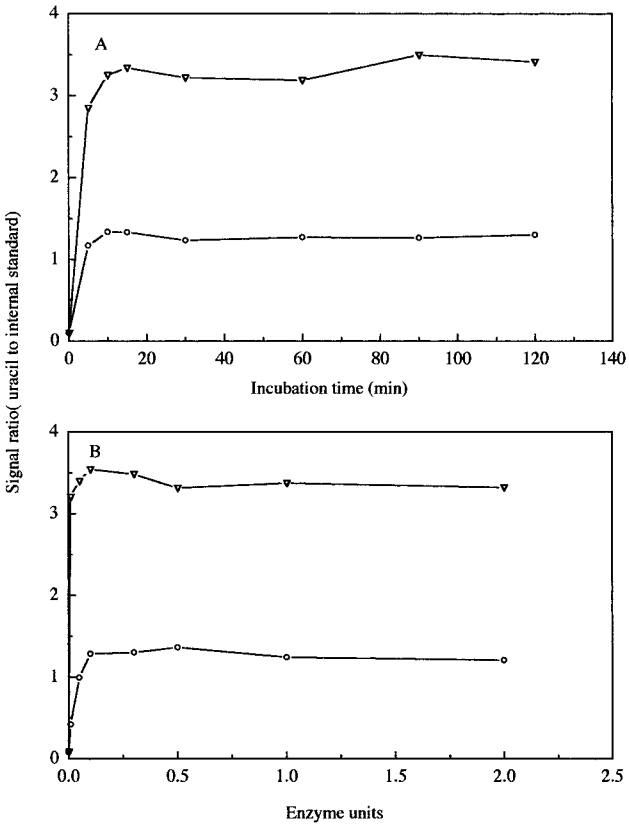

Figure 4. Effect of reaction time (upper panel, A) and enzyme concentration (lower panel, B) on uracil DNA glycosylase digestion of 10 μg of human DNA (circles) and 8.1 ng of PCR product (triangles) incubated at 37 °C. A 1-pm portion of $^{15}N_2$-uracil was added as an internal standard.

shown in Figure 3. In the remaining experiments, we used the parent → product ion combinations of 489 → 232 (uracil-MMB) and 491 → 233($^{15}N_2$-uracil-MMC).

DNA Glycosylase Digestion and Derivatization of Uracil. Uracil DNA glycosylase specifically removes uracil from DNA and is inactive on RNA.[12,13] We investigated the conditions of uracil DNA glycosylase digestion, and the results that we obtained are shown in Figure 4. The data demonstrate that the uracil formation is dependent on enzyme concentration and incubation time.

The reaction conditions of the uracil derivatization were optimized. We first examined pure uracil. The procedure was carried out at room temperature in nonaqueous media (acetone) using potassium carbonate as a base and 18-crown-6 as a phase-transfer catalyst.[22−24] Maximal yield was obtained after 40 min in the presence of 0.28 mmol/L BrMMC, 0.7 mmol/L 18-crown-6, and 5 mg of potassium carbonate.

With human DNA samples, however, we did not succeed in releasing uracil from the dried DNA samples. Vigorous shaking, ultrasonication, and the use of acetonitrile as a solvent failed; however, the addition of a minute volume of water (1.2 μL) to dried DNA before the derivatization enhanced the release of uracil. The interference from water with the anhydrous reaction conditions was prevented by increasing the amount of potassium carbonate, which may act by absorbing water. We observed that 1−4 μL water did not affect the derivatization of uracil in the presence of 40 mg of potassium carbonate.

Linearity, Detection Limit, and Precision. The uracil-MMC dissolved in 10 mM acetic acid in 50% acetonitrile was stable for at least 24 h at room temperature when kept in the dark.

Standards of uracil-MMC gave linear responses from 1.0×10^{-10} mol/L to 2.5×10^{-6} mol/L ($R = 0.999$). Using a signal-to-noise ratio of 3, the lower limit of detection was 1.0×10^{-10} mol/L. The data on precision of the assay are summarized in Table 1. The results demonstrate high reproducibility. Recovery was 95−106% (data not shown).

Table 1. Precision of the LC−MS/MS Method

	within-day ($n = 5$)		between-day ($n = 5$)	
sample[a]	U/DNA (mol/mol)	RSD (%)	U/DNA (mol/mol)	RSD (%)
1	8.0×10^{-6}	8.0	7.6×10^{-6}	10.0
2	51.0×10^{-6}	5.2	50.0×10^{-6}	7.2
3	0.14	1.2	0.14	5.0

[a] Sample 1 was human DNA, sample 2 was human DNA that had been heated at 95 °C for 10 min, and sample 3 was PCR product prepared as described in the method section.

Determination of Uracil in Human DNA. We used this assay to determine the uracil content of human DNA isolated from whole blood. Blood samples were obtained from subjects with normal folate status (red cell folate > 300 nmol/L) and from patients with folate deficiency (red cell folate <140 nmol/L) (Table 2). To check the reliability of the results, as a positive control, a known amount of PCR product with incorporated uracil was analyzed along with every set of samples. Our data (Table 2) shows that the uracil content of DNA isolated from whole blood did not differ according to folate status. Notably, the uracil content of human DNA obtained by this assay was lower than that

Table 2. Content of Uracil in DNA from Subjects with Normal or Impaired Folate Status

with folate deficiency					without folate deficiency				
subject	age years	sex	red cell folate nmol/L	U/DNA μmol/mol	subject	age years	sex	red cell folate nmol/L	U/DNA μmol/mol
D1	90	M	115	1.2	N1	60	F	782	6.0
D2	28	M	123	3.9	N2	55	M	432	9.6
D3	63	M	86	6.7	N3	42	M	508	4.8
D4	58	F	95	1.8	N4	37	M	365	8.0
D5	58	M	135	7.3	N5	52	M	622	6.5
D6	28	F	109	1.2	N6	30	M	376	6.6
D7	84	F	101	4.7	N7	45	F	404	5.7
D8	36	F	112	3.4	N8	56	M	581	2.9
D9	37	M	127	1.1	N9	47	F	402	5.2
D10	50	F	133	6.7	N10	52	F	580	9.1
D11	35	F	119	6.8	N11	38	F	480	3.2
D12	14	F	140	9.0	N12	37	F	317	6.6
D13	70	F	138	6.1	N13	27	M	405	1.6
D14	21	F	116	8.5	N14	35	M	504	6.8
D15	94	F	135	8.9	N15	43	M	375	1.0
D16	74	M	105	1.1	N16	47	F	884	1.8
mean			118	4.9				501	5.3
SD			16	3.0				157	2.6

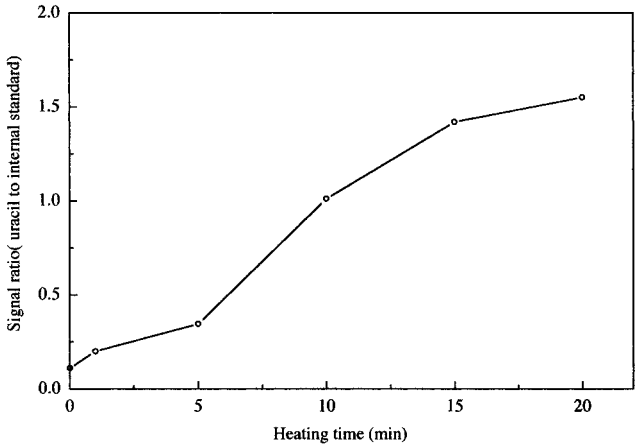

Figure 5. Formation of uracil from cytosine by deamination during heating of the DNA sample. Human DNA (10 μg) was first heated at 95 °C for 0−20 min and then incubated at 37 °C for 1 h in the presence of 0.3 unit uracil DNA glycosylase digestion. A 1-pm portion of $^{15}N_2$-uracil was added as an internal standard.

reported by others.[2,3] A different uracil content may be related to the different cell types that were investigated. Another possibility is different assay interferences from a coeluting material or the uracil derived from RNA or heated DNA. We tried to minimize such artifacts by combining the specificity of DNA uracil glycosylase and the selective detection afforded by tandem MS.

Deamination of Cytosine by Heat. The deamination of cytosine into uracil in DNA may occur in response to exposure to chemical mutagens.[5] In addition, it also occurs spontaneously when DNA is exposed to high temperature. We used the assay to examine the temperature-dependence of the cytosine deamination. The results are shown in Figure 5. Over a period of 20 min at 95 °C, the uracil content of human DNA increased about 40 times. This adds a cautionary note on interpreting reported uracil content in DNA. Our assay may be useful in the study of the chemistry of nucleic acids.

CONCLUSION

We have developed an assay based on HPLC−MS/MS for the determination of the uracil content in human DNA. Compared to other assays, the method is highly sensitive, fast, and specific, and it is nonradioactive. Furthermore, there is probably no interference from uracil from RNA because the DNA uracil glycosylase was used to specifically cleave uracil in DNA. The assay enabled us to measure the uracil content of human DNA that is assumed to be a result of misincorporation or cytosine deamination. Notably, we found that the uracil level did not differ between DNA isolated from whole blood of subjects with normal folate status when compared to those of impaired folate status. Notably, the uracil content of human DNA obtained in this assay is much lower than that previously reported in the literature.

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