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Gas chromatographic–mass spectrometric determination of 5-hydroxymethyluracil in human urine by stable isotope dilution

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ABSTRACT

A method for the determination of 5-hydroxymethyluracil in urine is described. 5-Hydroxymethyluracil was extracted by reversed-phase chromatography and quantified by gas chromatography–mass spectrometry as *tert.*-butyldimethylsilyl derivative. Since natural 5-hydroxymethyluracil contained *ca.* 22% of M + 2 species, an internal standard consisting of [1,3-$^{15}N_2$,5-$^{2}H_2$]hydroxymethyluracil was used to correct losses during extraction, evaporation and derivatization. Between-run precision of this method was 7.79%, and concentrations as low as 1.87 n*M* could be measured. This sensitivity and precision could not be obtained with trimethylsilyl derivatives.

INTRODUCTION

Living organisms produce free radicals, and this production may increase considerably in various conditions, including infections, reperfusion or exposure to near-UV and ionizing radiation. It has also been shown that free radicals lead to a broad range of harmful biological effects. Those concerning the nucleic acids are evidently the most severe for the individual and progeny. Al-

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terations of DNA by free radicals probably accelerate ageing [1,2] and promote cancer and mutagenesis [3–5]. The measurement of oxidized DNA bases in biological fluids can be used to study the clinical effects of free radicals on DNA, to indirectly monitor hydroxyl radical production, and to test the efficiency of antioxidant drugs.

Dizdaroglu [6,7] and Dizdaroglu and Bergtold [8] detected and characterized a large number of free radical-induced base products of calf thymus DNA with gas chromatography–mass spectrometry with selected-ion monitoring (GC–MS-SIM). Djuric *et al.* [9] used a similar technique, but with isotope dilution, to quantify 5-hydroxymethyluracil (HMU) in comparison with thymine residues in calf thymus DNA. To our knowledge, however, only a small number of methods are available for accurately quantifying nucleic acid adducts in biological fluids. Shigenaga *et al.* [10] and Park *et al.* [11] proposed a method based on high-performance liquid chromatography (HPLC) with electrochemical detection to measure urinary 7,8-dihydro-8-oxo-2′-deoxyguanosine. Although precise and selective, this method cannot be applied to most of the other oxidized bases or nucleosides. Bergtold *et al.* [12] quantitatively determined the thymine glycol/HMU ratio in animal and human urine by GC–MS [12].

This paper describes a GC–MS-SIM method that leads to the precise and specific quantitation of a DNA adduct, HMU, in urine. HMU, as thymine glycol, is derived from the attacks of hydroxyl radicals on thymine. Whereas thymine glycol is formed by saturation of the 5–6 double bond, HMU arises from hydrogen abstraction from thymine methyl by a hydroxyl radical [13,14].

EXPERIMENTAL

Chemicals

GC derivatives were prepared by using N-(*tert.*-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) (Fluka, Mullhouse, France).

Isotopically enriched HMU was synthesized from [1,3-$^{15}N_2$]uracil and [$^2H_2$]paraformaldehyde using the method of Cline *et al.* [15]. [1,3-$^{15}N_2$]Uracil and HMU were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was analytical-reagent grade (Prolabo, Paris, France) and water for HPLC was filtered and deionized through a Milli-Q water purification system (Millipore, Milford, MA, USA).

HPLC apparatus

For semipreparative HPLC a Kontron HPLC system (Kontron Analytic, Montigny le Bretonneux, France) consisting of two Model 420 pumps equipped with a Kontron 460 autosampler and a 450 data system to control pump flow and record the detector signal was used. The detector was a Shimadzu SP6AV UV (Shimadzu Electronics, Tokyo, Japan). Urine samples were purified on a 250 mm × 7.5 mm I.D. stainless-steel column packed with Spherisorb 5-μm ODS2. A 30 mm × 4.6 mm I.D. column packed with 10-μm RP18 mounted before the semipreparative column served as guard column. The column was eluted under isocratic conditions with water at a flow-rate of 2.5 ml/min. Then the column was recycled with acetonitrile–water (60:40, v/v). The components of the mobile phase were first filtered through Millipore 0.45-μm filters and degassed under vacuum. Fractions containing HMU were collected in 10-ml tubes in a Pharmacia FRAC-100 fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden).

GC–MS apparatus

A Nermag R-10-10 quadrupole mass spectrometer (Quad Service, Paris, France) was used in conjunction with a Nermag data system to calculate peak heights and plot chromatograms and spectra. Separations were performed on a Delsi SI200 gas chromatograph (Perkin Elmer France, Saint Quentin en Yvelines, France) equipped with a 25 m × 0.32 mm I.D. Chrompak CP Sil 5 column (Chrompak, Les Ullis, France) internally covered by a 1.2-μm film. Samples were injected with an all-glass falling-needle injector (Spiral, Couternon, Arc-sur-Tille, France) enabling volumes up to 5 μl to be injected.

Before derivatization, samples were evaporated to dryness with a Rotary Evapo-Mix (Buchler Nuclear Chicago Corporation, Fort Lee, NJ, USA).

HPLC extraction of HMU from urine

HMU was extracted from urine by semipreparative HPLC. The detection wavelength was 260 nm and the sensitivity 0.08 a.u.f.s. After precisely determining the retention time of the HMU peak (Fig. 1) by injecting 150 μl of a 5 mg/l standard, we collected the corresponding fraction in urine samples, which contained HMU.

Next, 5 ml of each urine sample were filtered through 0.45-μm Millipore filters and spiked with 20 μl of a solution containing 4.30 mg/l [1,3-$^{15}N_2$,5-$^{2}H_2$]HMU (solution 1, internal standard). The volume of the spiked urine injected was 150 μl, and each urine sample was injected ten times for a total volume of 1.5 ml per sample, which was found to be sufficient for the precise determination of HMU by GC–MS.

GC–MS measurement of HMU

Derivatization. The urine fractions obtained by HPLC and the standards were evaporated to dryness under vacuum using an Evapo-Mix at 40°C. The volume evaporated never exceeded 5 ml per tube (to avoid losses of HMU). The standards consisted of 1.5 ml of solutions at 14.1, 35.2, 70.4, 140.7 and 351.9 n*M* containing the oxidized base. Each standard solution was spiked with the same amount of [1,3-$^{15}N_2$,5-$^{2}H_2$]HMU as the urine samples (18 μl of a one third dilution of solution 1 in purified water per 1.5 ml). The residues were then redissolved in acetonitrile, and each urine sample and standard was collected in a single tube. The contents of each tube were evaporated at 70°C under a nitrogen stream. To the dry residue, 200 μl of a 25% (v/v) mixture of acetonitrile–MTBSTFA were added. Derivatization was then carried out by heating at 120°C for 60 min.

GC–MS measurements. The *tert.*-butyldimethylsilyl (t-BDMS) derivatives of standards and of urine extracts were analysed in the electron ionization mode. The electron energy was 70 eV, and the source temperature was set at 210°C. The injector temperature was 250°C, and the interface temperature was 280°C. The initial temperature of the GC oven was 150°C, and this was increased at 7°C/min to 290°C. Helium was used as the carrier gas and the pressure at the column inlet was 0.5 bar. Derivative volumes of 2–5 μl were desiccated in the injector and injected into the GC column. Peaks were quantified by considering only the mass fragments 427 (HMU) and

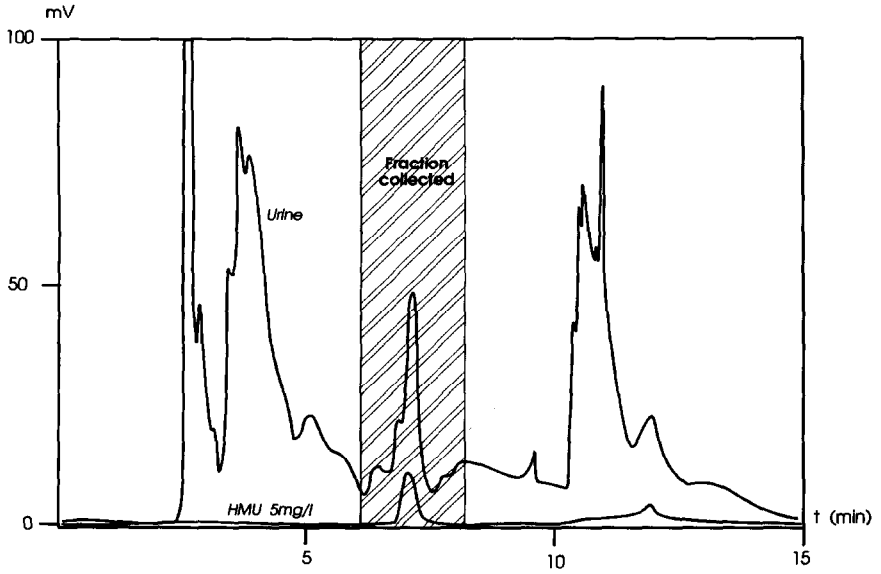

Fig. 1. Extraction of 5-hydroxymethyluracil from urine using semipreparative HPLC; 150 μl injected, detection wavelength 260 nm.

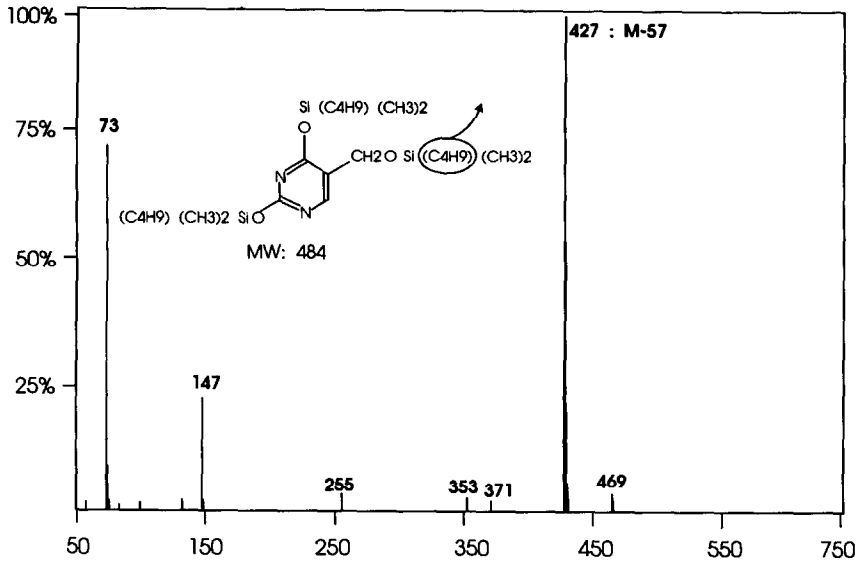

Fig. 2. Mass spectrum of the t-DBMS derivative of 5-hydroxymethyluracil obtained with a standard solution (1 mg/l).

431 ([1,3-$^{15}N_2$,5-$^{2}H_2$]HMU), which eluted at 17.75 min.

RESULTS AND DISCUSSION

An M + 4 labelled internal standard was used, because natural HMU was found to contain a high percentage (22%) of an M + 2 species that hampers exact determination of HMU in urine samples.

As previously described [6,16], t-BDMS derivatives provide an intense and characteristic $[M - 57]^+$ ion that is very useful for SIM measurements. The mass spectra of HMU (Fig. 2) and [1,3-$^{15}N_2$,5-$^{2}H_2$]HMU (Fig. 3) contain this peak, formed by the loss of a C_4H_9 group from the

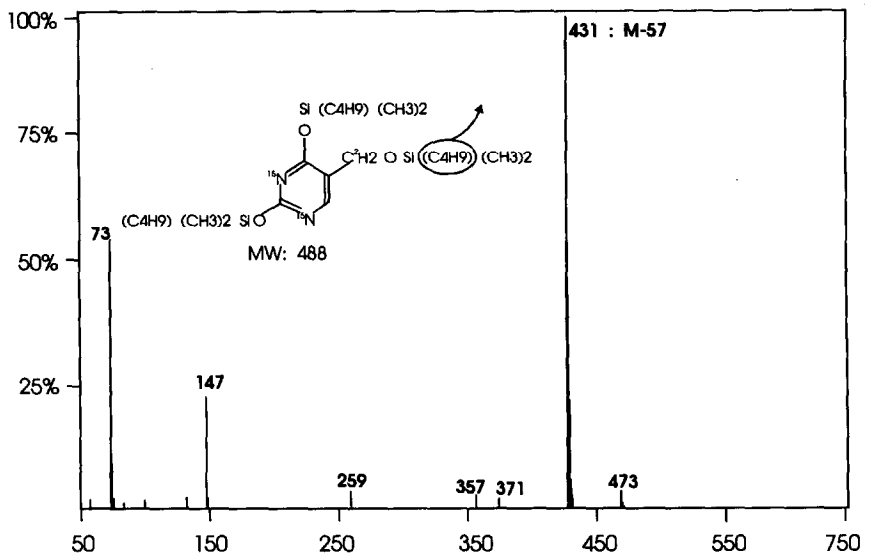

Fig. 3. Mass spectrum of the t-DBMS derivative of [1,3-$^{15}N_2$,5-$^{2}H_2$]hydroxymethyluracil obtained with a standard solution (1 mg/l).

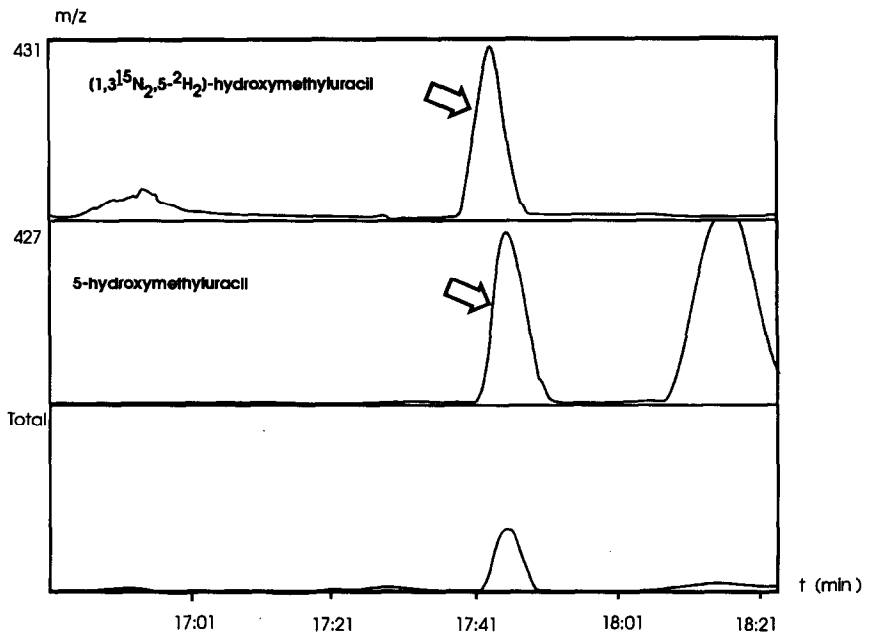

Fig. 4. Typical GC–MS-SIM chromatograms of a urine sample (58.4 n*M*).

tri-derivatized molecule of HMU. This ion was used to monitor HMU (*m/z* 427) and [1,3-$^{15}N_2$,5-$^{2}H_2$]HMU (*m/z* 431) in urine samples. The 427/431 ion area ratios on chromatograms (Fig. 4) were used to draw the standard calibration curve and subsequently to find urinary HMU concentrations. In addition to the characteristic M − 57 ion, the mass spectrum of HMU also contains a 469 ($[M - 15]^+$) ion arising from the loss of a methyl group. The peak of this ion was much less intense than the 427 peak and so it was not used to quantify HMU. Nevertheless, the 469 ion may be useful for calculating the 427/469 ratio, used for the unequivocal identification of HMU, but with a lower sensitivity. It should be noted that ions at *m/z* 429 and 430 are present in appreciable amounts (22.2 and 6.93% of the 427 ion, respectively). This is the reason for choosing [1,3-$^{15}N_2$,5-$^{2}H_2$]HMU as the labelled internal standard. However, a 1.2% contribution of the 427 ion to the 431 peak still occurs; the areas of the 431 peaks were corrected for this contribution.

Using this labelled HMU, the precision of the assay was determined (n = 10) by injecting the same standard solution (10 μl) derivatized separately in ten different vials. Under these conditions, the within-run precision, expressed as the coefficient of variation (C.V.), was found to be 5.62%. The between-run precision, measured in the same manner on two different days (n = 20), was found to be 5.55%. The within-day precision for urine samples containing 60.07 nmol/l HMU was found to be 6.06% (n = 9), and the between-run precision was 7.79% (n = 15) and, at the 51.6 n*M* level, the within-run precision was 8.85% (n = 8). The detection limit for HMU was 10 pg (signal-to-noise ratio of 4). The calibration curve of 427/431 corrected ratios as a function of concentrations of HMU was linear in the range 0–704 n*M* (r = 0.9932, twenty measurements).

Two different derivatizing reagents were compared: MTBSTFA and bis(trimethylsilyl)trifluoroacetamide (BSTFA). It was found that the t-BDMS derivative provided at least four-fold higher sensitivity than the tri-trimethylsilyl derivative (10 *versus* 40 pg). This finding is consistent with the results of Djuric *et al.* [9], who found that the detection limit for the trimethylsilyl derivative of HMU was 60 pg. Moreover, t-BDMS derivatives also led to a much better precision than did the trimethylsilyl derivative. For these

TABLE I

URINE EXCRETION OF 5-HYDROXYMETHYLURACIL IN TEN HEALTHY SUBJECTS

Subject	HMU in urine (nmol/24 h)	HMU (nmol/kg/24h)	HMU (nmol/mmol of creatinine)
1	158	2.47	11.3
2	104	2.08	9.63
3	74.0	1.54	7.30
4	108	2.07	9.47
5	200	3.22	13.9
6	153	2.18	10.4
7	306	4.08	12.3
8	221	3.40	12.7
9	135	2.14	14.1
10	115	2.30	10.9
Mean	157	2.55	11.2
S.D.	68.7	0.77	2.12

reasons MTBSTFA was chosen as the derivatizing reagent.

Urinary excretion of HMU in ten healthy volunteers, expressed as nmol/24 h, nmol/kg/24 h and nmol/mmol creatinine (Table I), shows that HMU daily excretion varies within a large range in normal subjects: however, a larger population is required to determine reference values. Bergtold *et al.* [12] determined the HMU/thymine glycol ratio as 0.5, and reported a value of 0.59 ± 0.29 nmol/kg/24 h for the normal excretion of thymine glycol. A simple calculation yields 82.6 ± 40.6 nmol/day for HMU excretion by an individual weighing 70 kg. Ames and Saul [17] also measured HMU excretion in urine, and reported an average of 62 nmol/day for an organism containing $6 \cdot 10^{13}$ cells. Although somewhat higher, the values listed in Table I are consistent with these data.

More work is required to determine whether the only source of HMU is DNA repair or if an appreciable portion of the daily excretion of this modified base arises from t-RNA, as suggested by Bergtold *et al.* [12].

CONCLUSION

The reagent used to determine HMU was MTBSTFA. As previously reported [6,16], the resulting derivatives lead to higher sensitivity and precision. Using this technique, assays can be accurately performed in 1.5-ml urine samples. The ease of collection of urine samples and the relative rapidity of the HMU assay make the procedure highly suitable for clinical studies. It should also be useful to test the efficiency of free radical protectors [18] and to search for side-effects of drugs known for their ability to generate free radicals.

The method can be also used for accurate measurement of HMU in dihydropyrimidine dehydrogenase deficiency [19], an inborn error of pyrimidine metabolism that results in the excretion of HMU at levels about 1000 times higher than in healthy people. This hyperecretion of HMU is associated with large increases in the excretion of thymine and uracil.

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