Detection and identification of 6-methylmercaptopo-8-hydroxypurine, a major metabolite of 6-mercaptopurine, in plasma during intravenous administration

CARIN W. KEUZENKAMP-JANSEN,1,2* JOHN M. VAN BAAL,1,2 RONNEY A. DE ABREU,1,2 JAN G.N. DE JONG,2 RICHARD ZUIDERENT,2 and J.M. FRANS TRIJBELS2

6-Mercaptopurine, a hypoxanthine antimetabolite, is used in the treatment of acute lymphoblastic leukemia (ALL) in children. Extensively metabolized before it exerts cytotoxic action, it is catabolized into 6-mercaptopo-2,8-dihydroxypurine (thiouric acid), which is excreted by the kidneys. We describe a metabolite of 6-mercaptopurine, 6-methylmercaptopo-8-hydroxypurine, whose presence has not been previously reported in plasma. This compound was found in high concentrations in plasma during high-dose 6-mercaptopurine infusions (1300 mg/m² in 24 h). This previously unknown compound was identified by reversed-phase HPLC with absorbance detection and by gas chromatography-mass spectrometry. The pathways leading to 6-methylmercaptopo-8-hydroxypurine in vivo are not yet fully understood. In a group of 17 patients treated with four courses of high-dose 6-mercaptopurine infusions according to the ALL-8 treatment protocol of the Dutch Childhood Leukemia Study Group, the steady-state concentrations of 6-methylmercaptopo-8-hydroxypurine in plasma were one-fifth of the parent drug concentrations, with wide interindividual variation. The formation of high concentrations of 6-methylmercaptopo-8-hydroxypurine in plasma, especially during the infusion, probably indicates another catabolic pathway of high-dose 6-mercaptopurine, apart from its conversion into thiouric acid.

INDEXING TERMS: acute lymphoblastic leukemia • methotrexate • drug metabolism • thiouric acid

1 Center for Pediatric Oncology SE Netherlands, 2 Laboratory of Pediatrics and Neurology, St. Radboud University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.
*Author for correspondence. Fax 31-24-6060426.
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Nonstandard abbreviations: 6MP, 6-mercaptopurine; 6MeMP(R), 6-methylmercaptopurine (riboside); 6MBOHP, 6-methylmercaptopo-2,8-dihydroxypurine; 6MeMBOHP, 6-methylmercaptopo-8-hydroxypurine; ALL, acute lymphoblastic leukemia; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl; and TPMT, thiopurine methyltransferase.
The presence of this metabolite of 6MP in plasma has not been described before and may point to another catabolic pathway of high-dose 6MP.

**Patients and Methods**

**Patients**

Patients with ALL (n = 17) were treated in our center according to the treatment protocol of the Dutch Childhood Leukemia Study Group (ALL 8 Study). They received four courses with high-dose methotrexate infusion (5 g • m⁻² in 24 h, from 0 to 24 h) followed immediately by a high-dose 6MP infusion (1300 mg • m⁻² in 24 h, from 24 to 48 h). Plasma was sampled before and at 24, 28, 42, 48, 52, and 72 h after the start of the methotrexate infusion. Informed consent was obtained from the patients or their parents according to the guidelines of the ethical committee of our hospital.

**Materials**

Calibrators of 6MP, 6-MP riboside, 6MeMP, 6MeMPR, thio- xanthine, and methylthioxanthine were obtained from Sigma Chemical Co., St. Louis, MO. Thiouric acid and methylthiouric acid were synthesized as described [11]. 6MeM80HP, 6MeM801HP, and 6-methylsulfinyl-8-hydroxypurine were provided by Gertrude B. Elion, Wellcome Research Labs., Research Triangle Park, NC.

**Procedures**

HPLC. HPLC was carried out as described [11]. In short, plasma was extracted with perchloric acid on ice and neutralized to pH 6–7 with K₂HPO₄. The metabolites were separated by reversed-phase HPLC with a 250 × 4.6 mm (i.d.) column of Supelcosil LC-18-DB (particle size 5 μm; Supelco, Bellefonte, PA). The mobile phase (flow rate 1.25 mL/min) consisted of a gradient from 0 to 25 min of two buffers, starting with 98:2 (by vol) buffer A (25 mmol/L KH₂PO₄) and buffer B (3 volumes of 50 mmol/L KH₂PO₄ plus 1 volume of methanol) and changing to 20:80 buffer A:buffer B; the latter conditions were maintained until 45 min after sample injection. Eluting analytes were detected with a variable ultraviolet-visible absorbance detector (Spectra Focus 2000 H/R system; Thermo Separation Products, Fremont, CA). For routine measurement the wavelengths were set at 290 and 320 nm; occasionally, the spectra of the peaks were scanned between 250 and 350 nm [11].

GC-MS. The unknown compound was collected from plasma by HPLC. To form the trimethylsilyl (TMS) derivative, we dissolved the isolated and lyophilized material in 50 μL of an equimolar mixture of chloroform and N,O-bis(trimethylsilyl) trifluoroacetic acid containing 10 mL/L trimethylchlorosilane (Pierce, Rockford, IL). We carried out the derivatization at 60 °C for 30 min, after which we diluted the mixture with 50 μL of chloroform. The 6MeM80HP calibrator was trimethylsilylated by the same procedure and used as a reference. To separate the products, we used a HP5890 gas chromatograph (Hewlett-Packard, Amsterdam, The Netherlands), using a 25 m × 0.32 mm (i.d.) CP-sil-8CB column with a film thickness of 0.12 μm (Chrompack, Middelburg, The Netherlands) and split injection. The carrier gas was helium at a column head pressure of 48.3 kPa. The oven temperature was programmed from 70 °C to 280 °C.

The metabolite of 6MP was identified with a VG-trio-2 quadrupole mass spectrometer (Fisons Instruments, Cheshire, UK) in electron impact ionization mode at 70 eV and a source temperature of 200 °C. Scan measurements were performed from 40 to 650 amu with a scan time of 1 s and an interscan delay of 0.1 s. Selected ion recording measurements were performed at the specific ions 254 (M⁺ - 1 TMS) and 326 (M⁺, di-TMS derivative) by using a span of 0.4 amu, a dwell time of 0.08 s, and an interchannel delay of 0.02 s.

**Statistics**

Statistics for 6MP and 6MeM801HP plasma concentrations were performed with the Software Package for the Social Sciences (SPSS). Descriptive statistics at each time point were calculated for each course of treatment. A paired r-test (95% confidence interval) was performed at each time point to compare the concentrations reached during successive courses. When the paired r-tests did not reach significance (P < 0.05), the concentrations of 6MP and 6MeM801HP reached during successive courses were not significantly different. We also calculated the individual means of the four courses for each patient at each time point, from which we calculated the descriptive statistics for the four courses together.
**Results**

On the basis of their retention time and ultraviolet absorbance, we determined that none of the calibrators described in Materials could account for the unknown peak. In HPLC, the unknown compound in plasma eluted at 29 min, i.e., 1.5 min before 6MeMP (Fig. 2A). When we added 1.8 \( \mu \)mol/L 6MeM8OHP to the plasma, the peak of the unidentified compound at 290 nm increased (Fig. 2A). In a different mobile phase, starting with 75:25 (by vol) buffer A:B and changing to 25:75 (by vol) buffer A:buffer B at 25 min, the unknown compound eluted at 24 min, as did 6MeM8OHP (Fig. 2B).

Moreover, the absorbance spectra of the unknown compound and of 6MeM8OHP were identical (Fig. 3).

The derivatized form of the 6MeM8OHP calibrator showed a peak with a retention time of 22.8 min by GC, the mass spectrum of which showed an abundant molecular ion at \( m/z \) 326 (M\(^+\)) and specific ions at \( m/z \) 311 (loss of CH\(_3\)), \( m/z \) 254 (loss of TMS), and \( m/z \) 239 (loss of TMS and CH\(_3\)). The chromatogram of the isolated and derivatized unknown compound showed a peak with a retention time of 23.0 min by GC with a mass spectrum identical to that of the derivatized 6MeM8OHP calibrator (Fig. 4, top panels). Selected-ion re-
recording measurements at the masses m/z 326 and 254 for both compounds showed retention times of 22.8 and 23.0 min for the derivatized calibrator and the derivatized unknown compound, respectively. The ratio between the ions was comparable (0.12 and 0.19, respectively) (Fig. 4, lower pairs of panels).

The areas under the peak of the unknown compound in the patients' samples were registered from the chromatograms at 290 nm, the wavelength at which 6MeMP and 6MeMPR were measured. The concentrations of the unknown compound were then calculated in retrospect. When the unknown compound was identified, we made calibration curves at 290 nm for 6MeMP, which eluted 1.5 min after 6MeM80HIP, and 6MeM80HIP. The correlation between the calibration curves of 6MeM80HIP (y) and 6MeMP (x) yielded the equation y = 0.9468x + 68.9 nmol/L. The concentrations of the unknown compound were first calculated from the areas of the unknown compound at 290 nm and the calibration curves of 6MeMP at 290 nm, which we had available from all series of HPLC measurements. These results (R1) were corrected for the difference between the two calibration curves: 6MeM80HIP concentration = (R1) - 0.9468 + 68.9 nmol/L.

We found no significant differences in the concentrations of 6MP or 6MeM80HIP reached during the successive courses at 28, 48, 52, or 72 h (P = 0.159–0.994, paired t-tests, 15–17 pairs). The minimum, median, and maximum concentrations of 6MP and 6MeM80HIP in 17 patients during the four treatment courses are indicated in Fig. 5. The concentrations of 6MeM80HIP were about one-fifth of those of the parent drug. The median interindividual CV during the 6MP infusion was 39% (range 6–118%) for 6MeM80HIP and 28% (range 1–132%) for 6MP. 6MeM80HIP was not detectable in urine—neither during the infusion nor in the next 24 h.

**Discussion**

This study provides strong evidence for the presence of 6MeM80HIP in plasma during and after high-dose 6MP infusions. The mass spectra of 6MeM80HIP and of 6-methylthioxanthine (6-methylmercapto-2-hydroxypurine) might be identical, but HPLC excluded the possibility that the unknown compound was 6-methylthioxanthine, showing a retention time of 18.7 min for the latter compound and different absorbance spectra.

To our knowledge, the presence of 6MeM80HIP in plasma has not been described before. A metabolite of 6MeMP described in urine of one patient accounted for 0.5% of the excretion of orally administered 6MeMP (6f) and was probably 6MeM80HIP. In our study, plasma 6MeM80HIP concentrations were about one-fifth of the parent drug concentrations,
both of which displayed wide interindividual variation. For 6MeM8OH, part of this variation may be caused by the wide interindividual variation in thiopurine methyltransferase (TPMT; EC 2.1.1.67) activity. TPMT shows a genetic polymorphism, with 88.6% of the subjects demonstrating high activity and 11.1% intermediate activity. About 1 in 300 subjects has undetectable TPMT activity [12].

TPMT activity is highest in liver and kidney but has been detected in all other tissues examined (erythrocytes, lymphocytes, thrombocytes, lymphoblasts, lung, intestine, brain, and placenta) [13]. How 6MeM8OH is formed in vivo is not known. Two metabolic routes may lead to the formation of this compound: methylation of 6M8OH or 8-oxidation of 6MeMP. Evidences exist from in vitro studies that oxidation of 6MP by xanthine oxidase preferentially occurs first at the 8 position and then at position 2, in contrast to hypoxanthine, which is first oxidized on C-2 and subsequently on C-8 [4]. 6M8OH has not been described in vivo, which may be explained by a higher activity of xanthine oxidase towards 6M8OH than towards 6MP [4]. We did not find 6M8OHIP in plasma or urine of the 17 patients. However, plasma of two patients treated in a therapeutic window phase with one high-dose 6MP infusion and with the xanthine oxidase inhibitor allopurinol [14, 15] contained a peak at 320 nm with the same retention time as 6M8OHIP, i.e., 1 min ± 6 s before the peak of 6MP. This peak was present during and after the 6MP infusion, and the area under the peak was 20–31% of that of 6MP in one patient (6MP steady-state 57 μmol/L) [14] and 6–11% in the other (6MP steady-state 35 μmol/L) [15]. We received the 6M8OHIP calibrator only recently from Dr. Elion. However, no more plasma from these two patients is available for analysis, so we cannot confirm that this peak was actually 6M8OHIP. The presence of this peak in the chromatograms of plasma of two patients with high 6MP steady-state concentrations and allopurinol treatment—and the absence of it in all chromatograms of the 17 patients treated with high-dose 6MP without allopurinol—suggest that 6M8OHIP can be produced in vivo and is rapidly further oxidized by xanthine oxidase into thiouric acid.

Recently, Deininger et al. demonstrated a $V_{\text{max}}/K_m$ ratio of 16.9 for TPMT with 6M8OH as substrate ($K_m$ 96.1 ± 2.3 μmol/L), whereas that with 6MP substrate was only 2.34 ($K_m$ 383 ± 7.0 μmol/L), indicating that 6M8OHIP is a better substrate than 6MP for TPMT [16]. Thus, 6MeM8OHIP might
be produced by methylation of 6Me8OH. On the other hand, 6Me8OH might also be produced by oxidation of 6MeMP. In vitro studies have shown that the relative oxidation rate of 6MeMP (relative to that of purine) was 15% for aldehyde oxidase (aldehyde:oxygen oxidoreductase purified from rabbit liver, EC 1.2.3.1) and <3% for xanthine oxidase (xanthine: oxygen oxidoreductase purified from bovine milk, EC 1.2.3.2) [17]. In our experience, xanthine oxidase (xanthine:oxygen oxidoreductase from buttermilk, EC 1.1.3.22; Sigma) converted only ~10% of 6MeMP into 6Me8OH in 4 h. Studies showing that thiouric acid is the main catabolite of 6MP in vivo [5–8, 10], and in vitro data demonstrating that oxidation of 6MP occurs preferentially first on C-8 [4] and that the Vmax/Km ratio for TPMT is higher with 6MOH as substrate than with 6MP [16], suggest that 6Me8OH may well be produced by methylation of 6MOH. Enzyme kinetic studies of xanthine oxidase and aldehyde oxidase (which is mainly active in the liver) with (methyl)thiopurine substrates are needed to elucidate the pathway leading to 6Me8OH. Given the wide interindividual variation of TPMT [12], it is important to know whether this enzyme acts to methylate 6MOH, which is a catabolite of 6MP, or 6MP, which is available for the anabolic pathway leading to cytotoxicity.

We treated nine patients with non-Hodgkin lymphoma at diagnosis with one high-dose 6MP infusion within a therapeutic window. Four patients received oral allopurinol and five did not. Plasma concentrations of 6MP, thioxanthine, and 6MeMP were higher and those of thiouric acid lower in the allopurinol-treated patients than in those who did not receive allopurinol [15]. The present study shows that 6Me8OH concentrations were higher in the group treated with allopurinol (3.7–17 μmol/L) than in those with no allopurinol (1.1–2.9 μmol/L). The higher concentrations of 6Me8OH in the allopurinol group may be the result of the higher concentrations of 6MP and 6MeMP in this group [15], the allopurinol-induced inhibition of further oxidation of 6M8OH, or the involvement of xanthine oxidase in any further metabolism of Me8OH.

The absence of 6Me8OH in urine of our patients might be explained by glucuronidation or further oxidation into 6-methylthioauric acid or 6-methylsulfinyl-8-hydroxypurine. 6Me8OH glucuronide accounted for 12–20% of the administered 6MeMP dose in urine [6]. In urine of two patients treated either orally or intravenously with 6-[18S]MP, considerable amounts of 6-methyl-[18S]sulfinyl-8-hydroxypurine and some 18Sulfate were excreted [7]. In a patient treated with 6-methyl-[18S]MP, 36% of the administered dose was excreted in urine as 6-methyl-[18S]sulfinyl-8-hydroxypurine and 27% as radioactive sulfate [6]. In humans, desulfuration of the thiopurine with formation of inorganic sulfate appears to occur via the methylthiopurines, which yield a larger amount of sulfate than the thiopurines do [7]. Because desulfuration of 6MP is not affected by allopurinol [18], xanthine oxidase probably is not involved in desulfuration of the methylthiopurines.

In conclusion, the present study shows that 6Me8OH is a major metabolite of 6MP in plasma during high-dose 6MP infusions, whereas smaller amounts of 6MeMP, 6MeMPR, and thioxanthine are produced in plasma [19]. The metabolic pathway leading to the formation of 6Me8OH or the further metabolism of this catabolite is not completely solved. Measurement of the 8-hydroxylated metabolites of 6MP in plasma and urine during high-dose 6MP infusions, as well as enzyme kinetic studies for xanthine oxidase and aldehyde oxidase with (methyl)thiopurine substrates, must be performed before we can obtain better insight into the catabolism of 6MP (apart from its conversion into thiouric acid) and the role of TPMT in the detoxification.

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