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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-mercapto-2,8-dihydroxypurine (thiouric acid), which is excreted by the kidneys. We describe a metabolite of 6-mercaptopurine, 6-methylmercapto-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-methylmercapto-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-methylmercaptopurine 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

*Nonstandard abbreviations: 6MP, 6-mercaptopurine; 6MeMP(R), 6-methylmercaptopurine (riboside); 6MeMBOHP, 6-methylmercaptopo-2,8-dihydroxypurine; 6MeM8OH1P, 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 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, thioxanthine, and methylthioxanthine were obtained from Sigma Chemical Co., St. Louis, MO. Thiouric acid and methylthiouric acid were synthesized as described [11]. 6M01HP, 6MeM801HP, and 6-methylsulfinyl-8-hydroxyxypurine 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 HR system; Thermo Sepacation 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].

**STATISTICS**

Statistics for the four courses to compare the concentration reached during successive courses. When the paired t-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 μmol/L 6MeM80HP 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 6MeM80HP (Fig. 2B). Moreover, the absorbance spectra of the unknown compound and of 6MeM80HP were identical (Fig. 3).

The derivatized form of the 6MeM80HP 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 CH₃), m/z 254 (loss of TMS), and m/z 239 (loss of TMS and CH₃). 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 6MeM80HP calibrator (Fig. 4, top panels). Selected-ion re-
concentrations of 0.9468 ± 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 FIPLC measurements. These results (R1) were corrected for the difference between the two calibration curves: 6MeM80HP concentration = (R1) - 0.9468 + 68.9 nmol/L.

We found no significant differences in the concentrations of 6MP or 6MeM80HP 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 6MeM80HP in 17 patients during the four treatment courses are indicated in Fig. 5. The concentrations of 6MeM80HP were about one-fifth of those of the parent drug.

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 6MeM80HP, and 6MeM80HP. The correlation between the calibration curves of 6MeM80HP (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: 6MeM80HP concentration = (R1) - 0.9468 + 68.9 nmol/L.

Fig. 3. Absorbance spectrum at 250–350 nm (top) and scan at the maximum at 29 min (bottom) of the unidentified compound in plasma (A) and of 6MeM80HP (B).

Discussion

This study provides strong evidence for the presence of 6MeM80HP in plasma during and after high-dose 6MP infusions. The mass spectra of 6MeM80HP and of 6-methylthiopurine (6-methylmercapto-2-hydroxypurine) might be identical, but HPLC excluded the possibility that the unknown compound was 6-methylthiopurine, showing a retention time of 18.7 min for the latter compound and different absorbance spectra.

To our knowledge, the presence of 6MeM80HP 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 [6] and was probably 6MeM80HP. In our study, plasma 6MeM80HP concentrations were about one-fifth of the parent drug concentrations,
Keuzenkamp-Jansen et al.: 6-Methylmercaptop-8-hydroxypurine

Fig. 4. (Upper pair of panels) Mass spectra of the 6MeM80HP calibrator and the unknown compound; (lower pair of panels) selected-ion recording (SIR) measurements of the 6MeM80HP calibrator and the unknown compound.

both of which displayed wide interindividual variation. For 6MeM80HP, 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 6MeM80HP is formed in vivo is not known. Two metabolic routes may lead to the formation of this compound: methylation of 6M80HP 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 [14]. 6M80HP has not been described in vivo, which may be explained by a higher activity of xanthine oxidase towards 6M80HP than towards 6MP [4]. We did not find 6M80HP 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 6M80HP, 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 6M80HP 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 6M80HP. 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 6M80HP can be produced in vivo and is rapidly further oxidized by xanthine oxidase into thiouric acid.

Recently, Deininger et al. demonstrated a $V_{max}/K_m$ ratio of 16.9 for TPMT with 6M80HP 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 6M80HP is a better substrate than 6MP for TPMT [16]. Thus, 6MeM80HP might
be produced by methylation of 6MeOHP. On the other hand, 6MeMOH 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) is 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 6MeMOHP in 4 h. Studies showing that thiouric acid is the main catabolite of 6MP in vivo [3–8, 10], and in vitro data demonstrating that oxidation of 6MP occurs preferentially first on C-8 [4] and that the V_{max}/K_m ratio for TPMT is higher with 6MOHP as substrate than with 6MP [16], suggest that 6MOHP may well be produced by methylation of 6MOHP. 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 6MOHP. Given the wide interindividual variation of TPMT [12], it is important to know whether this enzyme acts to methylate 6MOHP, 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 6MeMOH 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 6MeMOH 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 6M80HP, or the involvement of xanthine oxidase in any further metabolism of MeM80HP.

The absence of 6MeM80HP in urine of our patients might be explained by glucuronidation or further oxidation into 6-methylthiouric acid or 6-methylsulfinyl-8-hydroxypurine. 6MeM80HP 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-[^14]S|MP, considerable amounts of 6-methyl-[^14]Sulfinyl-8-hydroxypurine and some [14]Sulfate were excreted [7]. In a patient treated with 6-methyl-[^14]S|MP, 36% of the administered dose was excreted in urine as 6-methyl-[^14]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 6MeM80HP 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 6MeM80HP 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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