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

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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-mercaptop-2,8-dihydroxypurine (thioic 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-methylmercaptop-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-methylmercaptop-8-hydroxypurine in plasma were one-fifth of the parent drug concentrations, with wide interindividual variation. The formation of high concentrations of 6-methylmercaptop-8-hydroxypurine in plasma, especially during the infusion, probably indicates another catabolic pathway of high-dose 6-mercaptopurine, apart from its conversion into thioic acid.

INDEXING TERMS: acute lymphoblastic leukemia • methotrexate • drug metabolism • thioic acid
Statistics for the four courses to compare the concentrations of PDNS and 6MPR at each time point were calculated by the same procedure and used as a reference. To separate the products, we used a HP5890 gas chromatograph (Hewlett-Packard, Amsterdam, The Netherlands) 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+ IMS) and 326 (M+ di-IMS derivative) by using a span of 0.4 amu, a dwell time of 0.08 s, and an interchannel delay of 0.02 s.

**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]. 6M801H, 6MeM801H, 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 K₂HPO₄) and buffer B (3 volumes of 50 mmol/L K₂HPO₄ 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 Separation Products, Fremont, CA). For routine measurement, the wavelengths were set at 250 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) trifluoroacetamide 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 6MeM801H derivative 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+ IMS) and 326 (M+ di-IMS derivative) by using a span of 0.4 amu, a dwell time of 0.08 s, and an interchannel delay of 0.02 s.
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 6MMP (Fig. 2A). When we added 1.8 μmol/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-
...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 \( y = 0.9468x + 68.9 \text{ nmol/L} \). 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) \(-0.9468 + 68.9 \text{ nmol/L}\).

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 median interindividual CV during the 6MP infusion was 39% (range 6-118%) for 6MeM80HP and 28% (range 1-132%) for 6MP. 6MeM80HP 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 6MeM80HP in plasma during and after high-dose 6MP infusions. The mass spectra of 6MeM80HP 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 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 6MeM /6/ and was probably 6MeM80HP. In our study, plasma 6MeM80HP concentrations were about one-fifth of the parent drug concentrations,
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However, plasma of two patients treated in a high-dose 6MP without allopurinol—suggest that 6M80HP can be produced in vivo and is rapidly further oxidized by xanthine oxidase inhibitor allopurinol.

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 [4]. 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, 6M80HP might
be produced by methylation of 6M80HP. On the other hand, 6MeM80HP might also be produced by oxidation of 6MeMP. In vivo 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 6MeM80HP 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 6M80HP as substrate than with 6MP [16], suggest that 6MeM80HP may well be produced by methylation of 6M80HP. 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 6MeM80HP. Given the wide interindividual variation of TPMT [12], it is important to know whether this enzyme acts to methylate 6M80HP, 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, xanthine, 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 6MeM801HP 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 6MeM801HP 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.

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, 6MMPR, and thiourine 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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References


