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 (thiouric acid), which is excreted by the kidneys. We describe a metabolite of 6-mercaptopurine, 6-methylmercaptop-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 thiouric acid.

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

6-Mercaptopurine (6MP) is a hypoxanthine antimetabolite used in the treatment of acute lymphoblastic leukemia (ALL). 6 It has no intrinsic cytotoxic activity, but is converted into active metabolites before it exerts its cytotoxic action. The first step in the anabolic pathway of 6MP is its conversion into the nucleoside, thioinosine monophosphate (Fig. 1). This compound is converted into thioinosine monophosphate, which is cytotoxic after incorporation into DNA and RNA [11], or into methylthioinosine monophosphate, which is an inhibitor of the purine de novo synthesis [21]. 6MP can be methylated into 6-methylmercaptopurine (6MeMP). 6MeMP riboside (6MeMPR) is the product of the breakdown of methylthioinosine monophosphate or of the methylation of 6MP riboside. Xanthine oxidase (EC 1.2.3.2), which is mainly active in the liver and kidney [3], catalyzes 6MP into thiouric acid (6-mercaptop-2,8-dihydroxypurine). This oxidation occurs via 6-mercaptop-8-hydroxypurine (6MBOHP) and to a lesser extent via thioxanthine (6-mercaptop-2-hydroxypurine) [4]. Previous studies in humans demonstrated that 6MP and thiouric acid are the major compounds, and thioxanthine and 6MP riboside the minor ones, that are excreted in urine during 6MP administration [5–10], indicating that degradation of 6MP occurs mainly via xanthine oxidase. Another pathway leading to the inactivation of 6MP in humans is desulfuration of the drug, which probably occurs via methylthiopurines [7].

When treating patients with high-dose 6MP infusions, we found considerable amounts of a hitherto unknown compound in plasma [11]. After purification, the compound was identified by HPLC and gas chromatography–mass spectrometry (GC-MS) as 6-methylmercaptop-8-hydroxypurine (6MBOHP).
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, thio­xanthine, and methylthioxanthine were obtained from Sigma Chemical Co., St. Louis, MO. Thiouric acid and methylthiouric acid were synthesized as described [11]. 6M801HP, 6MeM801HP, and 6-methylsulfinyl-8-oxopurine 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 IR 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) 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 6MeM8OHHP derivative was trimethylsilylated by the same procedure and used as a reference. To separate the products, we used a HP5980 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⁺ – 1TMS) and 326 (M⁺, d1-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 t-test (95% confidence interval) was performed at each time point to compare the concentrations 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.

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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 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 CH1), m/z 254 (loss of TMS), and m/z 239 (loss of TMS and CH1). 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-
Table 1. Measurements of 6-MP and 6-MeMHOHP in plasma during and after high-dose 6-MP infusions. The minimum, median, and maximum concentrations of 6-MP and 6-MeMHOHP were about one-fifth of those of the parent drug. The median interindividual CV during the 6-MP infusion was 39% (range 6–118%) for 6-MeMHOHP and 28% (range 1–132%) for 6-MP. 6-MeMHOHP 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 6-MeMHOHP in plasma during and after high-dose 6-MP infusions. The mass spectra of 6-MeMHOHP and of 6-methylthioguanine (6-methylmercapto-2-hydroxypurine) might be identical, but HPLC excluded the possibility that the unknown compound was 6-methylthioguanine, showing a retention time of 18.7 min for the latter compound and different absorbance spectra.

To our knowledge, the presence of 6-MeMHOHP in plasma has not been described before. A metabolite of 6-MeMHP described in urine of one patient accounted for 0.5% of the excretion of orally administered 6-MeMHP [6] and was probably 6-MeMHOHP. In our study, plasma 6-MeMHOHP concentrations were about one-fifth of the parent drug concentrations.
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 [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 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 $V_{\text{max}}/K_m$ 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
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]SMP, considerable amounts of 6-methyl-[14]Sulfynyl-8-hydroxypurine and some [14]Sulfate were excreted [7]. In a patient treated with 6-methyl-[14]SMP, 36% of the administered dose was excreted in urine as 6-methyl-[14]Sulfynyl-8-hydroxypurine and 27% as radioactive sulfate [6]. In humans, desulfuration of the thiourine with formation of inorganic sulfate appears to occur via the methylthiopurines, which yield a larger amount of sulfate than the thiourines 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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