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Detection and identification of 6-methylmercapto-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-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/m^2 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-methylmercapto-8-hydroxypurine in plasma were one-fifth of the parent drug concentrations, with wide interindividual variation. The formation of high concentrations of 6-methylmercapto-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).[3] 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 nucleotide, thioinosine monophosphate (Fig. 1). This compound is converted into thioguanosine monophosphate, which is cytotoxic after incorporation into DNA and RNA *[1]*, or into methylthioinosine monophosphate, which is an inhibitor of the purine de novo synthesis *[2]*. 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]*, catabolizes 6MP into thiouric acid (6-mercapto-2,8-dihydroxypurine). This oxidation occurs via 6-mercapto-8-hydroxypurine (6M8OHP) and to a lesser extent via thioxanthine (6-mercapto-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-methylmercapto-8-hydroxypurine (6MeM8OHP).

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[3] Nonstandard abbreviations: 6MP, 6-mercaptopurine; 6MeMP(R), 6-methylmercaptopurine (riboside); 6M8OHP, 6-mercapto-8-hydroxypurine; 6MeM8OHP, 6-methylmercapto-8-hydroxypurine; ALL, acute lymphoblastic leukemia; GC-MS, gas chromatography–mass spectrometry; TMS, trimethylsilyl; and TPMT, thiopurine methyltransferase.

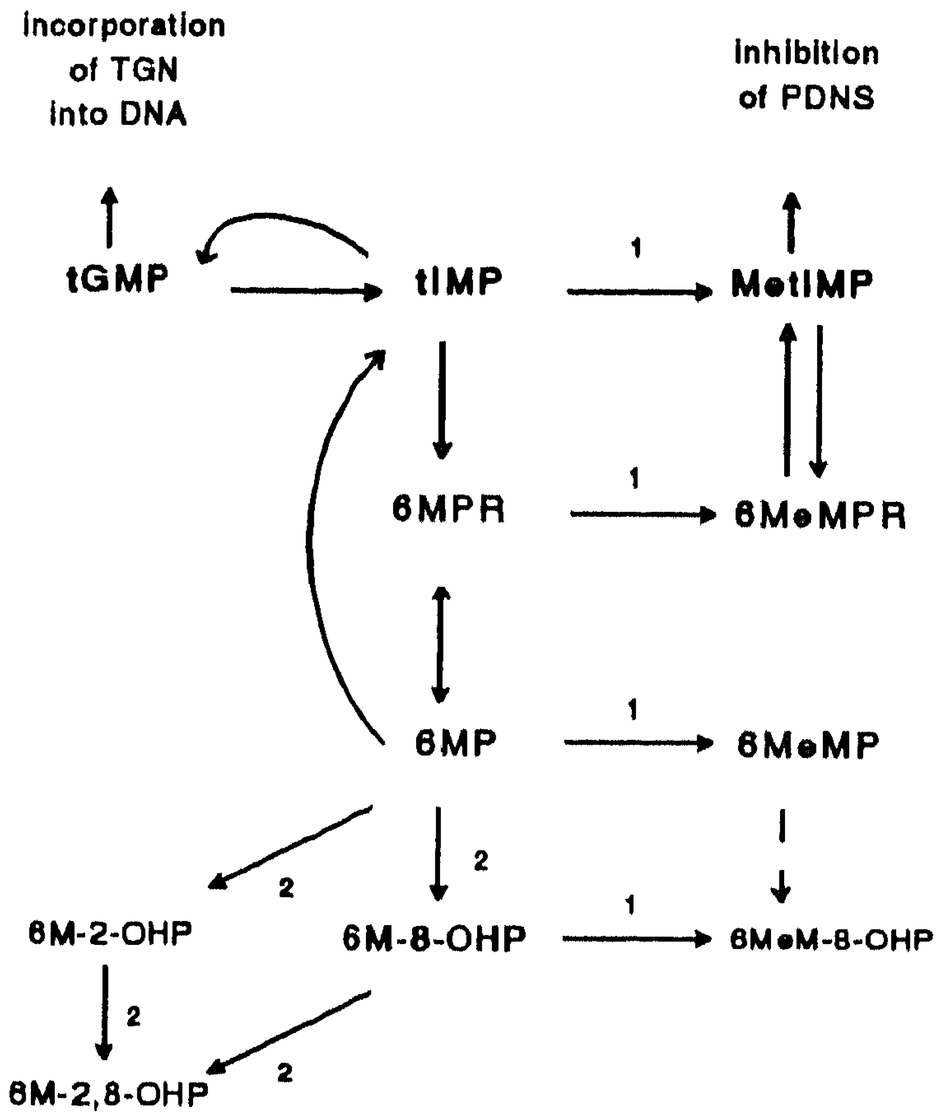

Fig. 1. Metabolism of 6MP.

(TGN) thioguanine nucleotides, (PDNS) purine de novo synthesis, (tGMP) 6-thioguanosine monophosphate, (tIMP) 6-thioinosine monophosphate, (MetIMP) 6-methylthioinosine monophosphate.For all other metabolites: Me = methyl, M = mercapto, OH = hydroxy, P = purine. (1) Thiopurine methyltransferase (EC 2.1.1.67), (2) xanthine oxidase (EC 1.2.3.2). The dotted lines represent pathways not definiteiy established.

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 $g \cdot m^{-2}$ in 24 h, from 0 to 24 h) followed immediately by a high-dose 6MP infusion (1300 $mg \cdot m^{-2}$ 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]*. 6M8OHP, 6MeM8OHP, 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 K_2HPO_4. 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 KH_2PO_4) and buffer B (3 volumes of 50 mmol/L KH_2PO_4 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 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 equivolume 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 6MeM8OHP 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^+ – 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 6MeM8OHP 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 ($P \leq 0.05$), the concentrations of 6MP and 6MeM8OHP 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.

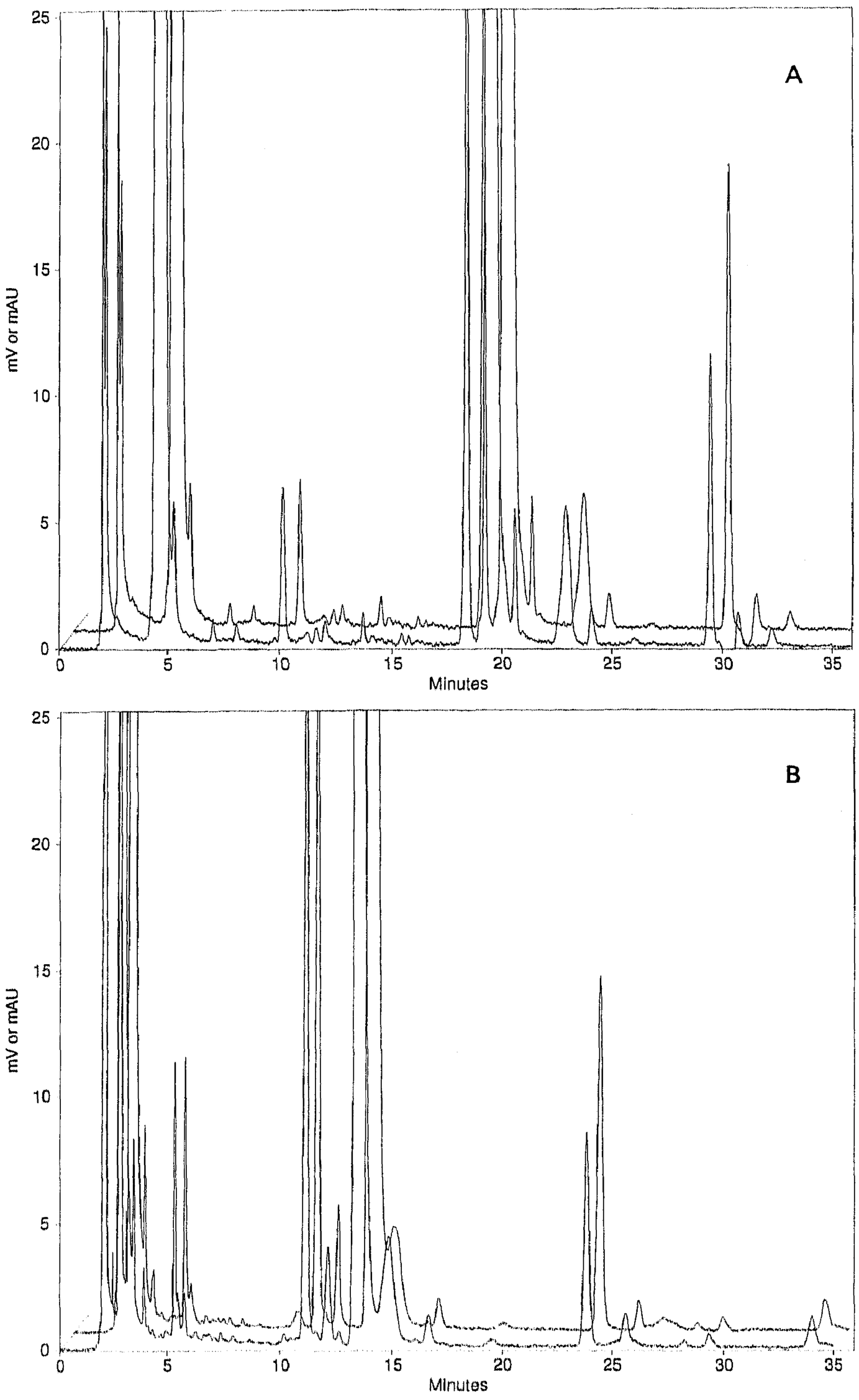

Fig. 2. Chromatograms at 290 nm of plasma sampled at 42 h after start of a methotrexate infusion of a patient treated with 24 h of high-dose methotrexate followed by 24 h of high-dose 6MP; 1.8 μmol/L 6MeM8OHP was then added to the plasma sample.

(*A*) Mobile phase as described in *Patients and Methods*, with the unknown compound and 6MeM8OHP eluting at 29.4 min and 6MeMP at 31 min. (*B*) Mobile phase as described in *Results*, with the unknown compound and 6MeM8OHP eluting at 24 min.

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 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-

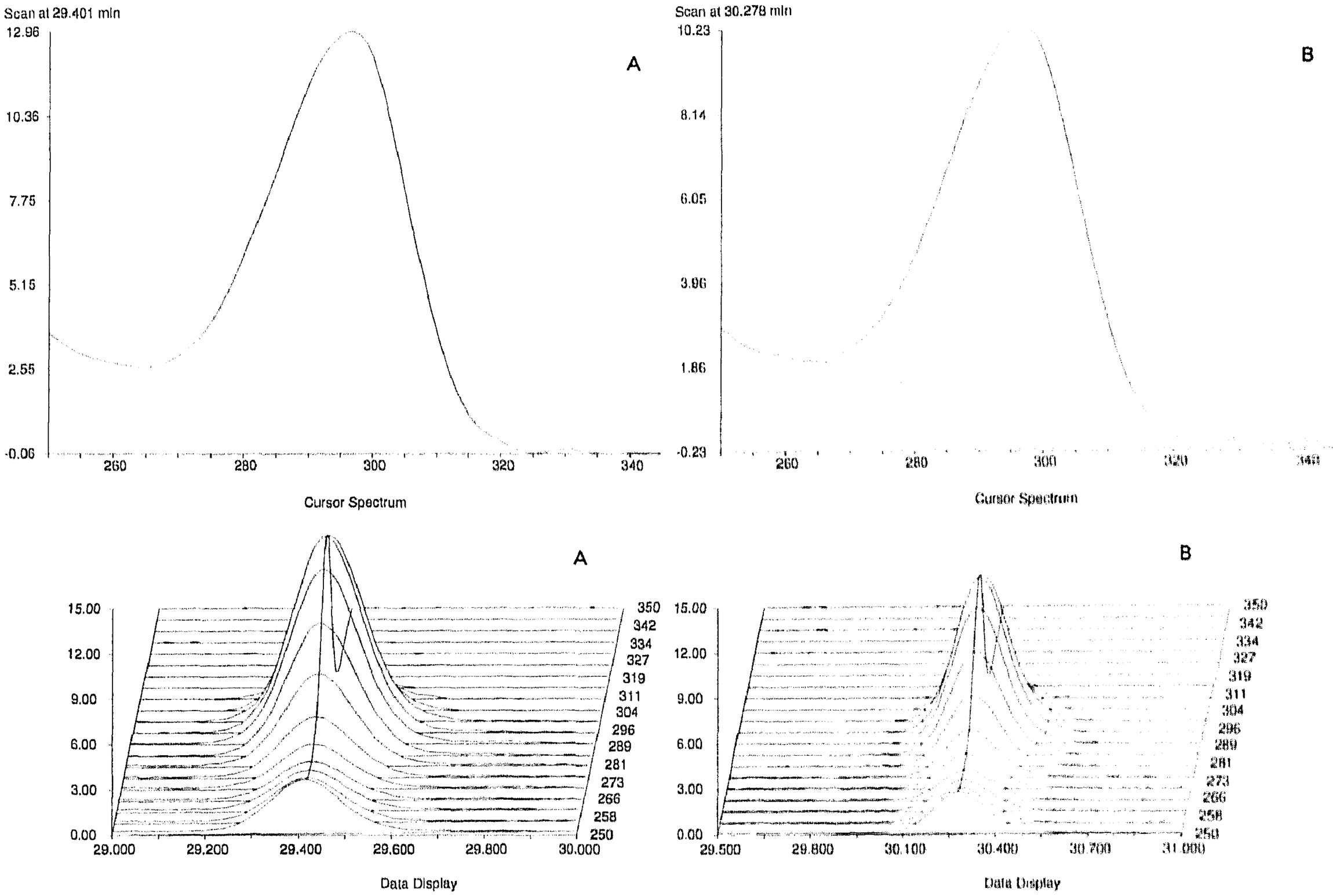

Fig. 3. Absorbance spectrum at 250–350 nm (*top*) and scan at the maximum at 29 min (*bottom*) of the unidentified compound in plasma (*A*) and of 6MeM8OHP (*B*).

cording 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 6MeM8OHP, and 6MeM8OHP. The correlation between the calibration curves of 6MeM8OHP (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: 6MeM8OHP concentration = (R1) · 0.9468 + 68.9 nmol/L.

We found no significant differences in the concentrations of 6MP or 6MeM8OHP 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 6MeM8OHP in 17 patients during the four treatment courses are indicated in Fig. 5. The concentrations of 6MeM8OHP were about one-fifth of those of the parent drug. The median interindividual CV during the 6MP infusion was 39% (range 6–118%) for 6MeM8OHP and 28% (range 1–132%) for 6MP. 6MeM8OHP 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 6MeM8OHP in plasma during and after high-dose 6MP infusions. The mass spectra of 6MeM8OHP 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 6MeM8OHP 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 6MeM8OHP. In our study, plasma 6MeM8OHP concentrations were about one-fifth of the parent drug concentrations,

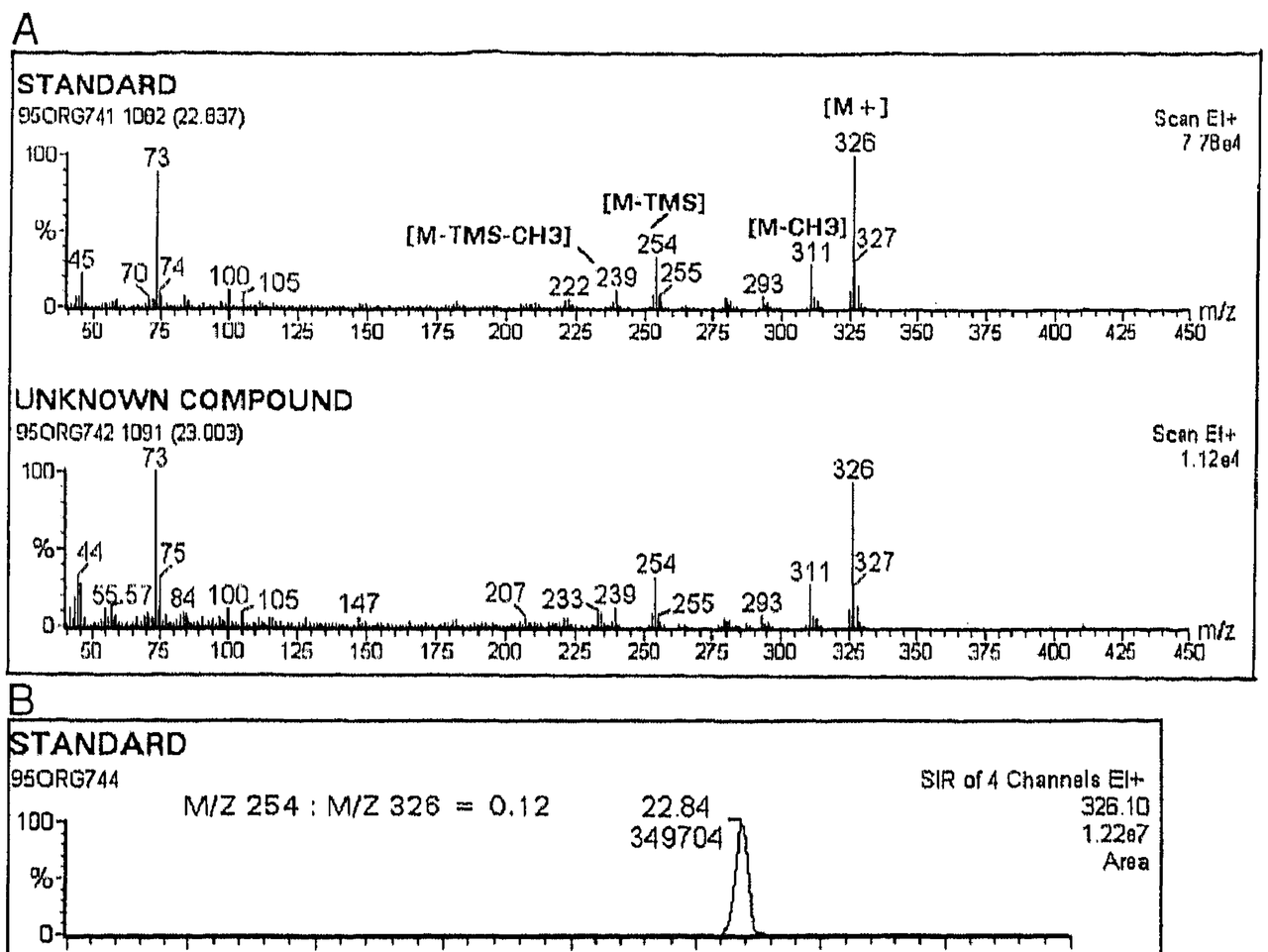

Fig. 4. (*Upper pair of panels*) Mass spectra of the 6MeM8OHP calibrator and the unknown compound; (*lower pairs of panels*) selected-ion recording (SIR) measurements of the 6MeM8OHP calibrator and the unknown compound.

both of which displayed wide interindividual variation. For 6MeM8OHP, 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 6MeM8OHP is formed in vivo is not known. Two metabolic routes may lead to the formation of this compound: methylation of 6M8OHP 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]*. 6M8OHP has not been described in vivo, which may be explained by a higher activity of xanthine oxidase towards 6M8OHP than towards 6MP *[4]*. We did not find 6M8OHP 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 6M8OHP, 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 6M8OHP 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 6M8OHP. 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 6M8OHP 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 6M8OHP 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 6M8OHP is a better substrate than 6MP for TPMT *[16]*. Thus, 6MeM8OHP might

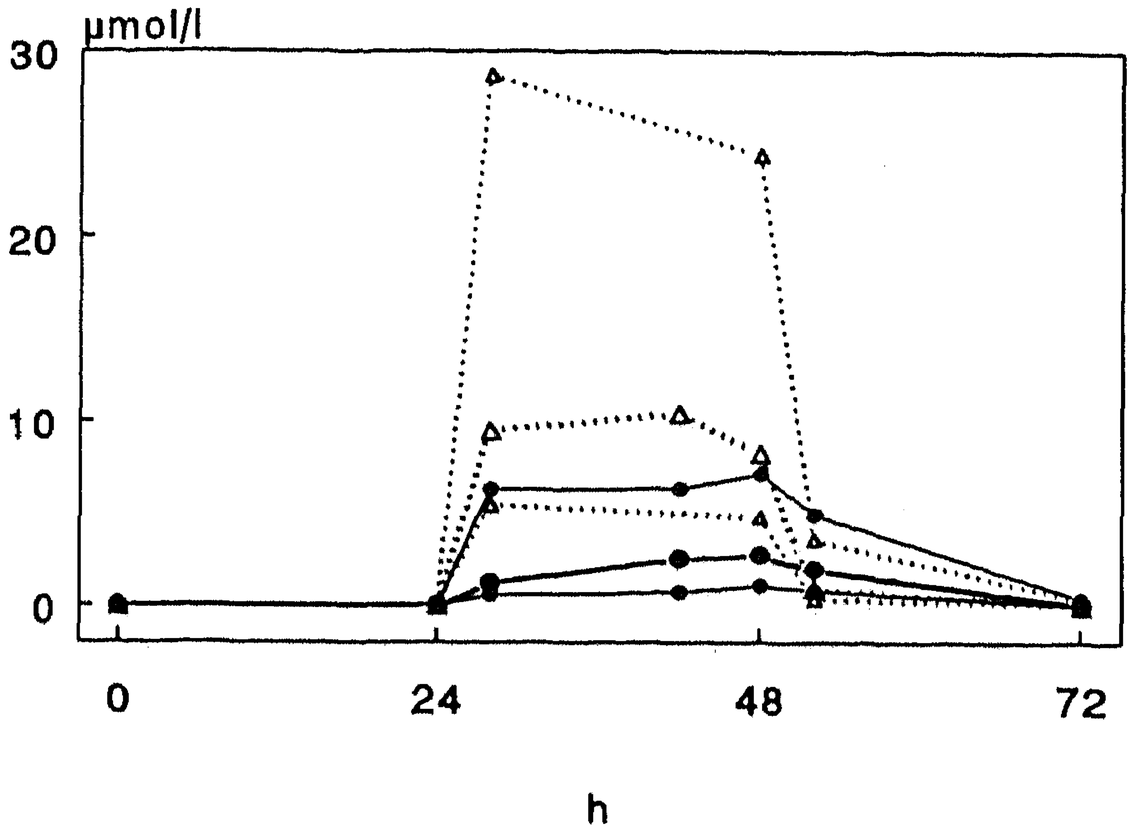

Fig. 5. Minimum, median, and maximum plasma concentrations of 6MeM8OHP (—) and 6MP (- - -) during four courses with high-dose methotrexate infusions (5 g · m^{-2} in 24 h) immediately followed by high-dose 6MP infusions (1300 mg · m^{-2} in 24 h) in 17 patients.

be produced by methylation of 6M8OHP. On the other hand, 6MeM8OHP 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 6MeM8OHP 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 V_{max}/K_m ratio for TPMT is higher with 6M8OHP as substrate than with 6MP *[16]*, suggest that 6MeM8OHP may well be produced by methylation of 6M8OHP. 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 6MeM8OHP. Given the wide interindividual variation of TPMT *[12]*, it is important to know whether this enzymes acts to methylate 6M8OHP, 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 6MeM8OHP 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 6MeM8OHP 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 6M8OHP, or the involvement of xanthine oxidase in any further metabolism of MeM8OHP.

The absence of 6MeM8OHP in urine of our patients might be explained by glucuronidation or further oxidation into 6-methylthiouric acid or 6-methylsulfinyl-8-hydroxypurine. 6MeM8OHP 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-[^{35}S]MP, considerable amounts of 6-methyl-[^{35}S]sulfinyl-8-hydroxypurine and some [^{35}S]sulfate were excreted *[7]*. In a patient treated with 6-methyl-[^{35}S]MP, 36% of the administered dose was excreted in urine as 6-methyl-[^{35}S]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 6MeM8OHP 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 6MeM8OHP 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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