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Note

Rapid method for evaluating compliance of 6-mercaptopurine therapy in children with leukemia*

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6-Mercaptopurine (6-MP), one of the oldest antineoplastic agents [1], is administered orally daily to prolong the duration of remission induced by other drugs in both acute and chronic leukemias [2]. Although the precise mechanism of action of 6-MP as an antileukemic drug is not fully understood, this purine analogue interferes with nucleic acid biosynthesis [3,4] by competing with hypoxanthine and guanine for the enzyme hypoxanthine-guanine phosphoribosyl transferase [5] and is itself converted to the active metabolite 6-thioinosinic acid (TIMP). The latter is methylated to form methylthioinosinate (MTIMP). Both TIMP and MTIMP have been reported to inhibit glutamine 5-phosphoribosyl pyrophosphate amido-transferase, the first enzyme in the pathway for purine ribonucleotide synthesis [6]. Following oral administration, 6-MP is absorbed incompletely [7–12]; less than 25% of the dose is absorbed in children [7,10].

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The absorption is attenuated by food [13]. 6-MP exhibits a short but variable plasma half-life (range 0.8–2.2 h [7,10,12]).

Some patients either do not respond to 6-MP or suffer a relapse during maintenance therapy [14]. Several putative mechanisms for maintenance therapy failure have been proposed: (a) development of resistance in tumor cells that have lost the capacity to convert 6-MP to TIMP [3,5,15]; (b) poor absorption after oral administration; (c) poor patient compliance, especially in children [16,17]; and (d) physician non-compliance with the recommended doses of chemotherapy [17,18]. Thus, "non-responders" may not be achieving therapeutic levels of the drug. A rapid and simple, preferably non-invasive, method was needed to determine compliance with 6-MP therapy.

Several methods have been described for the isolation and quantitation of 6-MP in serum, plasma and/or urine. Some are based on gas chromatography [19] or gas chromatography–mass spectrometry [20] of derivatized 6-MP, high-performance liquid chromatography (HPLC) [21–29], phosphorescence spectroscopy [30], spectrophotometry [31] and measurement of fluorescence of the oxidized [32] or derivatized [33,34] products of 6-MP. These procedures have one or more of the following disadvantages: (a) lengthy and complicated methodology; (b) low sensitivity and (c) use of potentially toxic solvents (e.g., dichloromethane) for extraction of 6-MP.

We developed a rapid and simple procedure for measurement of 6-MP in urine and plasma, involving solid-phase extraction of the drug followed by HPLC analysis. The procedure was applied, non-invasively by measurement of urinary drug levels, to assess compliance in pediatric patients on maintenance therapy with 6-MP.

EXPERIMENTAL

Materials

6-MP was obtained from Sigma (St. Louis, MO, U.S.A.). HPLC solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals of analytical grade were procured locally.

Instrumentation and operating procedures

A Waters Assoc. HPLC system equipped with a reversed-phase C_{18}, 10 μm, 30 cm$\times$0.4 cm column, a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and an ISCO (Omaha, NB, U.S.A.) variable-wavelength detector (set at 320 nm) were used for the assay. A mobile phase consisting of a degassed mixture of methanol–acetonitrile–0.005 *M* KH_2PO_4 (pH 4.0) (10:0.5:89.5, v/v/v) at a flow-rate of 1.5 ml/min was used as the eluent. Samples were analyzed at ambient temperature (approximately 22°C). Standard solutions of 6-MP (in water) were protected from light (by wrapping the containers in aluminum foil) and stored at or below 4°C; no significant degradation occurred over two months.

Sample collection

Urine samples were collected (by spontaneous voiding) from fourteen children (six females; 2–13 years old; Table I) undergoing therapy with 6-MP (50 mg/m^2 orally) as outpatients. The history of taking 6-MP and other medications (to detect interference in the HPLC assay) on that day and the previous two days was recorded but kept secret until after the assays. Control urine samples were also collected from other subjects not receiving 6-MP.

6-MP assay procedure

Standard solutions of 6-MP (1–50 μg/ml; in water) were analyzed by HPLC by using the above system; standard curves were constructed from the amounts of the drug injected (0.01–1.0 μg) onto the column and the peak heights obtained.

TABLE I

PATIENT PROFILE AND URINARY LEVELS OF 6-MERCAPTOPURINE

Patient No.[a]	Sex[b]	Age (years)	Weight (kg)	Diagnosis[c]	6-MP dose[d] (mg/day)	Other drugs co-administered[e]	Sample time[f] (h)	6-MP in urine (μg/ml)
1	F	3	13	ALL	25	M, T, S	>24	1.0
2	M	6	27	ALL	50	M, T, P	2–3	8.6
3	M	4	19	ALL	25/50	M, T, S	<12	9.2
3	M	4	19	ALL	25	M, T, S	>24	1.0
4	M	4	24	ALL-pre-B	25	M, T, S	>12	2.7
4	M	4	24	ALL-pre-B	25	M, T, S	48	0.8
5	M	5	21	ALL-N	25	M, T, S	>24	1.0
6	M	2	11	ALL	25/50	T, S, P	6–8	2.7
6	M	2	11	ALL	25/50	M	24	1.8
7	M	5	24	ALL-N	25	M, T, S	<12	7.9
8	F	6	22	ALL-pre-B	25	M, T, S	<12	6.9
9	F	5	22	ALL	25/50	M, T, S	<12	4.0
10	M	9	30	ALL	50	M, T, S	<12	5.5
11	F	3	15	ALL-N	25	M, T, S	72–96	<0.1
12	F	13	47	ALL	100	M, T, S	24	1.2
13	F	13	57	ALL	50/100	M, T, S	12–24	2.2
14	M	5	23	ALL	50	M, T, S	12–24	2.6
Controls (n=8)								<0.1

[a]Urine was collected from patients No. 3, 4 and 6 on two occasions.

[b]F = female; M = male.

[c]ALL = acute lymphoblastic leukemia; ALL-pre-B = acute lymphoblastic leukemia, pre-B cell; ALL-N = acute lymphoblastic leukemia, Null cells.

[d]All children received 6-mercaptopurine orally at a dose of 50 mg/m^2, once a day. Some children were maintained on the same daily dose continuously, while others received a lower and then the higher daily dose on alternative weeks.

[e]P = prednisone; T = trimethoprim; S = sulfamethoxazole; M = methotrexate. All drugs were administered orally except in patient No. 6 who received methotrexate by infusion.

[f]Approximate collection time of urine sample after the last dose of 6-MP as reported by the patient or the guardian.

Measurement of 6-MP in urine

Urine was filtered (0.22-μm Millipore filter) and aliquots (2–50 μl) were injected directly onto the column. To achieve higher sensitivity and specificity, 6-MP was isolated by solid-phase extraction prior to HPLC measurement as follows:

Sep-Pak cartridges (C_{18}, Waters Assoc.) were preconditioned by washing sequentially with water (2 ml), methanol (2 ml) and then water (2 ml). Blank urine (2 ml) was diluted with an equal volume of 0.2 *M* phosphate buffer (pH 7.4) containing increasing amounts of 6-MP (0–50 μg/ml). The diluted urine was applied to the preconditioned cartridges. The latter were washed with buffer (2 ml) and then eluted with methanol (2 ml). The methanol phase (5–200 μl) was analyzed by HPLC. Confirmation of the 6-MP peak in the chromatogram was carried out by superimposition by spiking the samples with known amounts of 6-MP prior to injection on the column.

For the measurement of 6-MP in patients' urine, aliquots (2 ml) were diluted with equal volumes of the phosphate buffer and applied to the preconditioned cartridges. The latter were extracted as above and the methanol phase (5–200 μl) was analyzed by HPLC. The recovery of 6-MP in each sample was determined by assaying duplicate aliquots of urine (2 ml) spiked with 8 μg of the drug.

Measurement of 6-MP in plasma

6-MP was assayed in plasma as follows: plasma, obtained from heparinized blood by centrifugation (20 min, 500 *g*, 4°C), was diluted 1:1 (v/v) with the phosphate buffer containing 1 mg/ml ascorbic acid. This mixture was applied to the preconditioned cartridges and the adsorbed 6-MP was extracted as above. The methanol phase was evaporated to dryness (nitrogen), the residue was reconstituted in 0.5 ml of the mobile phase and aliquots (5–200 μl) were injected on the column and analyzed as above.

RESULTS AND DISCUSSION

6-MP is widely used to maintain remission in acute and chronic leukemia. The usual maintenance regimen consists of daily oral doses of 6-MP and weekly oral doses of methotrexate. Despite uniform maintenance therapy, relapse of leukemia occurs in some patients. Some of the reasons for failure of drug regimen include (a) development of resistance to 6-MP, due to decreased conversion to the active species [3,5,15]; (b) poor absorption of the drug [7,8,10–13,35]; (c) variability in the disposition of 6-MP [12,36,37]; and (d) non-compliance [16,17]. Thus, non-responders may not be achieving therapeutic levels of the drug and its active metabolites in plasma and tissues due to pharmacokinetic reasons, enzyme deficiency or erratic dosage administration.

To help identify causes of non-response, we developed a simple and rapid HPLC method for measurement of 6-MP in urine and applied the procedure to assess compliance in patients on maintenance 6-MP therapy.

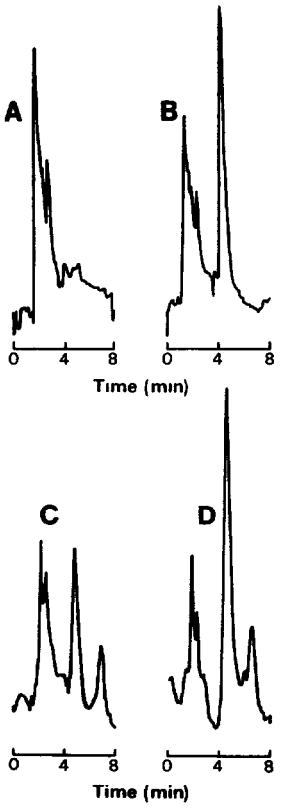

Fig. 1. Chromatograms obtained after injection of the methanol eluate of Sep-Pak cartridges treated with urine samples (see text). (A) 20 μl of eluate from blank urine; (B) 16 μl of eluate from blank urine spiked with 3.2 μg/ml 6-MP; the peak appearing at 5 min corresponds to 48 ng of 6-MP (recovery = 98%); (C) 5 μl of the eluate from urine of patient No. 10 showing 27 ng of 6-MP; (D) 4 μl of the eluate from the urine (patient No. 10) spiked with 8.0 μg/ml 6-MP.

6-MP eluted at 5.0 min; its peak was confirmed by superimposition after spiking the samples with known quantities of the drug. Detector response was linear for 0.01–1.0 μg 6-MP injected. Injection-to-injection variability was 4–5% and day-to-day variation was 6–8%. There were more background peaks in the chromatograms when urine was injected directly on the column; the limit of detection was 50 ng/ml 6-MP in urine. More than 95% of 6-MP in urine was recovered by the solid-phase extraction method (Fig. 1). The limit of detection of the extraction method was 20 ng/ml in urine. The lower limit of detection of the procedure can be improved by concentrating the methanol extract of the Sep-Pak cartridges and reconstituting the residue in a small volume of the mobile phase prior to HPLC assay. However, for measurement of non-compliance this is not necessary.

The extraction method was also applied to the measurement of 6-MP in plasma; the recovery was >95% and the limit of detection was 10 ng/ml 6-MP in plasma.

Drugs such as prednisone, trimethoprim, sulfamethoxazole and methotrexate taken by the patients along with 6-MP did not interfere in the assay.

A total of seventeen samples collected from fourteen patients were assayed by the procedure. Urine samples from all but one patient on 6-MP therapy showed the presence of significant amounts of the drug (Table I). The parent of this three-year-old patient (No. 11) admitted missing administration of the drug for three or four days. In addition, the urine of four other patients who had not taken their medication in the last 24 h prior to the clinic visit (i.e., non-compliants) had low levels of 6-MP (≤ 1.0 μg/ml).

The present method has the advantage over previous methods in that it does not involve tedious methodology and/or extraction of 6-MP with potentially toxic solvents and is non-invasive. It can be used as an outpatient procedure for surveillance of compliance in patients with leukemia receiving 6-MP.

CONCLUSIONS

We have developed a rapid, simple and non-invasive method for determining compliance in patients on maintainence therapy with 6-MP. The procedure can also be of use in identifying patients with poor absorption of the drug due to intrinsic causes or as a result of the presence of food in the stomach. The method is based on isolation of 6-MP from spot urine samples by solid-phase extraction followed by measurement of the drug by reversed-phase HPLC. Our studies detected that one third of the pediatric leukemic patients undergoing maintenance 6-MP therapy did not adhere to the recommended dosage regimen.

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