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Note

Sensitive and specific high-performance liquid chromatographic assay for 6-thiouric acid

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The success of therapeutic drug monitoring depends upon an understanding of the pharmacology and metabolism of a drug as well as the availability of a clinically useful analytical procedure for the measurement of the parent drug or metabolites. Both 6-mercaptopurine and azathioprine possess relatively little biologic effect as administered and must be anabolized into their active thiopurine nucleosides and nucleotides [1–3]. After administration, both parent drugs are quickly taken up by nucleated cells and disappear from the plasma. Azathioprine appears to serve primarily as a pro-drug for 6-mercaptopurine, which in turn is subjected to rapid conversion to its active intracellular metabolites. Because of the rapid disappearance of 6-mercaptopurine or azathioprine from the plasma, measuring blood or urine concentrations of these compounds alone has not proven useful for therapeutic drug monitoring [4,5]. Analysis of 6-thiouric acid (6TU), a 6-mercaptopurine metabolite which is present in both urine and plasma [6], may be used as a marker for the complex metabolism of azathioprine and 6-mercaptopurine [7]. Breter and Zahn [8] have shown a biphasic production of 6TU by cell cultures exposed to 6-mercaptopurine. An initial appearance of 6TU was seen within 1 h and a second increase in 6TU concentration began at 8 h and continued to rise at 24 h. This biphasic 6TU production may allow alternative metabolic pathways for the parent drug to be inferred [7].

Previously published analytical methods for 6-mercaptopurine [9,10] have not been designed for the measurement of 6TU. While the assay of Van Baal et al. [11] was capable of detecting 6TU, the lack of analytical-grade 6TU prevented

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the establishment of a 6TU calibration curve. Narang et al. [12] made no attempt to quantitate 6TU concentrations in plasma. Lennard [13] was unable to detect 6TU at concentrations less than 500 ng ml^{-1} using the described analytical conditions. A method for the measurement of 6TU in urine has been reported [14]; however, this procedure was able to detect 6TU only in the 5–50 mg l^{-1} range. For possible use in a program of therapeutic drug monitoring, any assay of 6TU must have adequate sensitivity, must be capable of separating 6TU from plasma components and other thiopurine metabolites, and must be relatively rapid. In this report, we describe a high-performance liquid chromatographic (HPLC) assay which has these characteristics.

EXPERIMENTAL

Instrumentation

The HPLC apparatus consisted of a Beckman Model 110A pump (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with an Altex Model 210 injection valve with a 100-μl sample loop. A guard column (7 cm × 2.2 mm I.D., Whatman) was packed with pellicular C_{18}-bonded silica particles, 20–40 μm in diameter (Whatman, Clifton, NJ, U.S.A.), and connected between the injection valve and the analytical column. The analytical column was a reversed-phase Ultrasphere ODS (Beckman) column, 25 cm × 4.6 mm I.D., packed with 5-μm diameter particles. The detector was a variable-wavelength UV–Vis detector (Hitachi Model 110-A) equipped with an Altex liquid chromatography flow cell. Detection was at 350 nm. The flow-rate for the mobile phase was 1.5 ml/min. Output from the detector was processed by a Hewlett-Packard Model 3390A integrator-chart recorder (Hewlett-Packard, San Diego, CA, U.S.A.) which was used in the peak-height mode.

Reagents and animals

6-Mercaptopurine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Azathioprine was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). Thioguanine, 6-methylmercaptopurine, 8-hydroxymercaptopurine and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). Analytically pure 6TU was prepared in our laboratory after the method of Levin et al. [15]. Acetonitrile (HPLC grade) was supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Glacial acetic acid was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). The water used to prepare all solutions and the mobile phase was double-distilled, deionized ASTM Type II water (Barnstead PCS purification system, Sybron, Boston, MA, U.S.A.). The mobile phase was acetonitrile–glacial acetic acid–water (20:2:78). Human plasma was obtained from the American Red Cross (Madison, WI, U.S.A.). The plasma was harvested from blood containing citrate–phosphate–dextrose anticoagulant. The animal used in these experiments was the C57/Bl6 male mouse (Harlan Sprague–Dawley, Madison, WI, U.S.A.). The mice were six weeks old and weighed 18–20 g.

Procedure

Calibration standards of 6TU in human plasma were prepared by weighing out the desired amount of the compound, dissolving it in the minimum amount of 1.0 *M* sodium hydroxide solution, and quickly diluting the sample with human plasma. In all cases the minimum ratio of plasma to sodium hydroxide solution was 100:1. The calibration standards were divided into aliquots of 0.3 ml in 4.0-ml glass screw-cap vials. A 10-μl volume of a 0.33 *M* dithiothreitol solution was added to each vial to protect the sulfhydryl groups of the thiopurine compounds. The calibration standards were frozen at −70°C and stored at this temperature until used. The concentrations of 6TU in the calibration standards were 5000, 1000, 500, 250, 125, 50, 20, 10 and 0 ng ml^{-1}.

An internal standard solution of 6-thioguanine was prepared by stirring an excess of solid thioguanine in HPLC-grade acetonitrile for 24 h at 25°C in a water bath. This saturated solution was filtered and 75 ml of the solution were diluted to 1 l with additional acetonitrile and mixed well. This solution was divided into 20-ml aliquots and stored at 4°C until needed. Spectrophotometric analyses of the internal standard solutions showed no detectable decomposition of the thioguanine throughout assay development.

At the time of chromatographic analysis, the vials containing the calibration standards were allowed to thaw; 1.0 ml of the thioguanine–acetonitrile internal standard solution was added to each vial. The vials were vortexed for 1 min. The acetonitrile precipitated the plasma proteins, which formed a semi-solid mass at the bottom of the vials. The contents of the vials were carefully transferred into plastic centrifugal filter tubes (Schleicher and Schull, Keene, NH, U.S.A.) which had a piece of filter paper (Whatman No. 50) installed between the compartments of the tube. The tubes were centrifuged at 1500 *g* for 10 min to filter the samples.

The resultant clear samples were then transferred with disposable glass pipets to new, disposable 10 mm×75 mm glass culture tubes. The liquid in the tubes was evaporated under a gentle stream of nitrogen at 30°C. The residue in the tubes was reconstituted with 100 μl of mobile phase. The tubes were sealed with Parafilm (American Can, Greenwich, CT, U.S.A.) to prevent evaporation. The tubes were vortexed twice for 45 s, and then centrifuged to assure that any liquid which had splashed onto the glass walls would be pulled back down to the bottoms of the tubes. The tubes were stored after reconstitution at room temperature in the dark until the contents were assayed. Standard curves were calculated using peak-height ratios of 6TU to the internal standard. Regression analysis was performed on each calibration curve. Statistical evaluation of the fitted regression line was performed as described by Natrella [16].

To establish the chromatographic identity of the metabolites of azathioprine (6-mercaptopurine, 8-hydroxymercaptopurine, 6-methylmercaptopurine, 6TU) as well as 6-thioguanine and dithiothreitol, a small amount of each compound was dissolved in mobile phase and injected individually into the HPLC system. The retention times observed for each known compound were matched with the peaks from the calibration standards.

To confirm the presence of 6TU in murine plasma following 6-mercaptopurine

administration, the mice were given 20 mg/kg 6-mercaptopurine by intraperitoneal injection. The mice were allowed free access to water after dosing. There were ten mice in each treatment group. Following drug administration, the mice were sacrificed at the following times, respectively: 0.25, 0.5, 1, 2, 3, 4, 8, 10 and 12 h. One mouse was studied as a control. At the appropriate time, the animal was rendered unconscious by diethyl ether. Blood was withdrawn from the vena cava into a syringe prefilled with 0.1 ml of a 0.13 *M* sodium citrate solution. The blood was transferred to a disposable plastic centrifuge tube and centrifuged to separate cells from plasma, then 0.3 ml of plasma were removed and placed in a glass screw-top vial and 10 μl of dithiothreitol solution were added to the plasma. Vials were stored at $-70\,^{\circ}$C until analysis. Murine plasma samples were prepared for analysis using the same procedure outlined for the calibration standards. The experiment was conducted three times.

RESULTS

The chromatographic peak-height ratio (6TU/internal standard) was proportional to 6TU plasma concentration, and the calibration curve was highly linear over the range 0–5000 ng ml^{-1} (correlation coefficient >0.9998). The limit of detection was 55 ng ml^{-1} when all nine standards ranging from 0 to 5000 ng ml^{-1} were used for the calibration curve. The limit of detection was 15.4 ng ml^{-1} when only the six standards ranging from 0 to 250 ng ml^{-1} were used. In either case, the limit of detection was taken to be the concentration of 6TU for which the predicted peak-height ratio for a single new measurement would be statistically greater than zero with 95% confidence when calibration curves derived from triplicate determinations of each standard were used. The coefficient of variation estimated from the triplicate measurements was less than 10% for 6TU concentrations above 319 ng ml^{-1} and was between 15 and 20% near the limit of detection.

A typical chromatogram obtained from human plasma spiked with 6TU is shown in Fig. 1. Retention times of the various compounds varied slightly with ambient temperature; however, the usual retention times for 6TU, 6-thioguanine (internal standard) and the dithiothreitol plasma complex were 4.5, 11.8 and 16.4 min, respectively. The retention times for 8-hydroxy-6-mercaptopurine, 6-mercaptopurine, 6-methylmercaptopurine were approximately 5.3, 6.2 and 8.8 min, respectively. Plasma samples containing all metabolites yielded chromatograms in which all peaks were well separated (Fig. 2).

The results of the mouse experiments are presented in Table I. These data show a rapid decline in plasma 6TU concentrations, with a nadir occurring at 6 to 8 h. 6TU concentrations begin to rise between 10 and 12 h reflecting the release of intracellular metabolites and their subsequent degradation to 6TU.

DISCUSSION

It is our belief that 6TU shows promise as a plasma and urinary marker for the biologic effects of azathioprine and 6-mercaptopurine [7]. While others have attempted to develop analytical methods for the parent drugs, we have modified

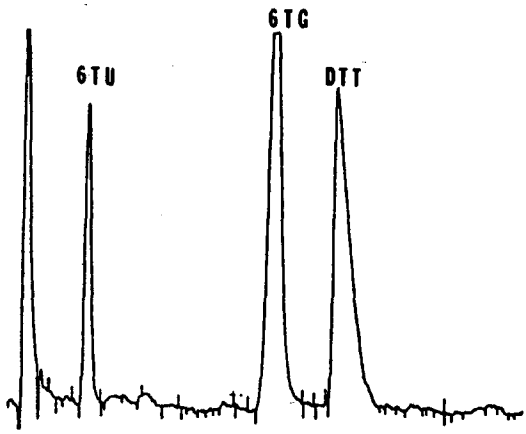

Fig. 1. Typical chromatogram obtained from human plasma spiked with 6-thiouric acid. Peaks: 6TU = 6-thiouric acid; 6TG = 6-thioguanine, internal standard; DTT = dithiothreitol.

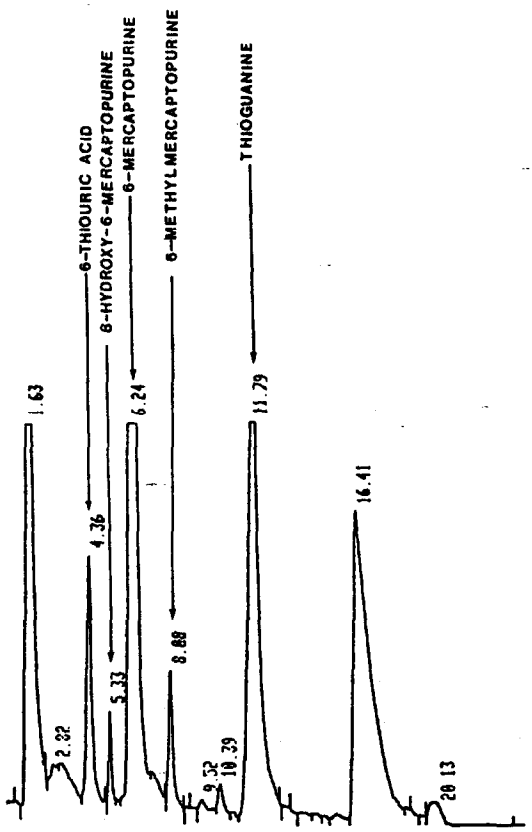

Fig. 2. Chromatogram of plasma containing several metabolites of azathioprine.

TABLE I

PLASMA CONCENTRATION OF 6TU AFTER TREATMENT WITH 6-MERCAPTOPURINE

Range indicates upper and lower 95% confidence limits.

Time post-dose (h)	Concentration (ng/ml)					
	Experiment I		Experiment II		Experiment III	
	Mean	Range	Mean	Range	Mean	Range
0.25	457.0	449.5-464.6	3451.3	3359.0-3547.8	2744.2	2593.0-2908.5
0.50	448.0	440.5-455.6	1178.5	1129.6-1228.9	450.0	367.9- 534.3
1	144.9	137.5-153.0	215.8	175.5- 256.5	114.1	104.9- 124.3
2	48.2	43.4- 53.2	76.2	65.4- 88.3	110.1	101.1- 120.0
3	33.4	28.8- 38.1	20.3	11.6- 29.5	35.2	28.5- 42.1
4	21.5	17.1- 26.1	28.1	19.3- 37.5	16.0	9.6- 22.6
6	8.3*		10.5*		10.7*	
8	9.1*		6.3*		0.0*	
10	11.1*		13.8*		6.8*	
12	31.4	26.8- 36.1	38.3	29.2- 48.1	29.3	22.8- 36.1

*Lower 95% confidence limit indistinguishable from 0.

previously published methods to develop a sensitive, specific and rapid assay for the separation of 6-mercaptopurine from some of its metabolites and for the measurement of 6TU. Previous attempts to measure 6TU [11,13,14] have lacked adequate sensitivity. The degree of sensitivity of our assay for detecting 6TU exceeds that of previous methods. It is anticipated that by using larger volumes of plasma, the sensitivity of this assay would be enhanced even further.

The rapid decline and subsequent rise in 6TU concentrations in plasma from intact animals occurred in agreement with previous cell culture work [8]. As can be seen from Table I, the secondary peak in 6TU concentration was not complete at 12 h after treatment with 6-mercaptopurine. Complete characterization of this peak requires sampling until all 6TU has disappeared from the plasma. With this observation in mind, future studies should include extended sampling times.

Because this analytical method is capable of detecting thiopurine metabolites, an application for the procedure will be in pharmacokinetic studies of azathioprine or 6-mercaptopurine. While its greatest value may be as a tool for probing the pharmacokinetics and pharmacodynamics of azathioprine and 6-mercaptopurine, this assay may also be used as a screening assay for 6TU to determine compliance with thiopurine therapy.

With one exception, the instruments and reagents required for this procedure are readily available. To our knowledge, analytical-grade 6TU may not be commercially available in the U.S.A. This may necessitate the synthesis of this compound [15]. The analytical methodology outlined in this report is well suited for most clinical laboratories experienced with HPLC.

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