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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF 6-THIOGUANINE APPLICABLE TO PHARMACOLOGIC STUDIES IN HUMANS

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SUMMARY

6-Thioguanine (6TG) and its metabolites were analyzed in human plasma with a reversed-phase high-performance liquid chromatographic method. 6TG and related compounds were extracted from plasma with an equal volume of 2 *N* perchloric acid at a 50—100% recovery efficiency. The neutralized extracts were chromatographed on a μBondapak C_{18} column by two separate isocratic conditions. 6TG, 6-thiouric acid, 6-thioxanthine, 6-thioguanosine, and 6-methylthiouric acid were analyzed with 0.01 *M* sodium acetate, pH 3.5—10% methanol as the mobile phase and 340 nm for detection. 6-Methylthioguanine and three unknown metabolites were separated with acetate—25% methanol and 310 nm detection. One of the unknowns was identified as 6-methylthioguanosine. External standard calibration was used for quantitation. The 6TG detection limit was 0.8 nmol/ml in plasma.

INTRODUCTION

6-Thioguanine (6TG) has been used as a cancer chemotherapeutic agent for several decades. The analytical techniques developed over the years for the pharmacokinetic study of this drug, however, are lacking for a number of reasons. An early method that separated metabolites on Dowex columns followed by spectrometric quantitation [1], is time consuming. The use of ^{35}S-labelled 6TG for detection and paper chromatography for separation of metabolites [2] involves the problems inherent in administration of radio-labelled compounds to humans. A spectrofluorometric assay following oxidation with hydrogen perioxide [3] suffers from both a lack of specificity for metabolites and poor sensitivity. Another method entailing oxidation to a fluorescent product with alkaline permanganate [4] followed by anion-exchange chromatography [5] does not allow detection of 6-thiouric acid (6TU),

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6-thioxanthine (6TX), or 6-methyl metabolites. An anion-exchange high-performance liquid chromatographic (HPLC) method [6,7] although suited for the analysis of one of the 6TG metabolites, 6-thioguanosine monophosphate, requires a long time to analyze each sample. A recent report has suggested that intermittent high dose 6TG may have utility in the treatment of colorectal carcinoma [8]. The initiation of this protocol prompted us to investigate a reversed-phase HPLC system for the routine and sensitive assay of 6TG and related compounds in patient plasma.

EXPERIMENTAL

Materials

6-Thioguanine (NSC-752) was obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). 6TX, 6-thioguanosine (6TGS) and guanosine monophosphate were obtained from Sigma (St. Louis, MO, U.S.A.). 6TU, 6-methylthioguanine (6MeTG), 6-methylthiouric acid (6MeTU), and 6-methylthioxanthine (6MeTX) were kindly provided by Dr. Gertrude Elion of Burroughs-Wellcome (Research Triangle Park, NC, U.S.A.). Acetate buffer (0.01 *M*) was made from glacial acetic acid (Mallinckrodt, St. Louis, MO, U.S.A.) and de-ionized water (Millipore, New Bedford, MA, U.S.A.), and the pH was adjusted with 5 *M* sodium hydroxide. This buffer was filtered (Millipore HA 0.45 μm) and degassed before the addition of degassed methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) to make the mobile phase. The pH 2.5 mobile phase was adjusted with 1 *N* hydrochloric acid. Oxidation to fluorescent species was carried out by the method of Finkel [4] using potassium permanganate (Baker reagent) and an SPF-125 spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.). 6TU, 6TX and 6MeTG could not be oxidized to fluorescent products by this method.

HPLC

A Spectra-Physics Model 3500B (Santa Clara, CA, U.S.A.) liquid chromatograph is used for solvent delivery. Samples are injected through a Valco 6-port rotary valve fitted with a 100-μl sample loop onto a 30 cm × 3.9 mm μBondapak C_{18} column (particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.). 6TG and metabolites are detected with an SP 770 variable-wavelength UV detector (Spectra-Physics) at a sensitivity of 0.04 a.u.f.s. Peaks are traced on a Hewlett-Packard 7130A strip chart recorder at 0.25 in./min using a 10 mV setting, and integrated with a Spectra-Physics Autolab System I Computing Integrator. Optimization of conditions was carried out with acetate buffer as the A solvent and methanol—acetate (1:1, v/v) as the B solvent. Two separate conditions are used for the detection of metabolites in clinical specimens. 6TG, 6TU, 6TX, 6TGS, 6MeTU and 6MeTX are eluted with a mobile phase of 0.01 *M* sodium acetate, pH 3.5—10% methanol (v/v) and detected at 340 nm. 6MeTG is eluted with 0.01 *M* sodium acetate, pH 3.5—25% methanol (v/v) and detected at 310 nm. Flow-rates are 2.0 ml/min and produce column pressures of 11.1 MPa and 13.8 MPa respectively. The column is flushed daily with methanol—water (1:1, v/v).

Extractions

Triplicate 1.0-ml plasma samples containing known concentrations of 6TG or metabolite standards were precipitated with 1.0 ml of cold 2 *N* perchloric acid (PCA), and centrifuged at 48,000 *g* at 4°C for 20 min. Aliquots (1.0 ml) of the resulting supernatants were removed, adjusted with 0.150 ml 4 *N* potassium hydroxide to pH 10—12, and stored at 4°C for two days to precipitate all of the perchlorate salts [9]. The solutions were adjusted to pH 2—3 with 0.150 ml of 1 *N* hydrochloric acid (to avoid dissolution of the silica support of the column) and centrifuged at 700 *g* at room temperature for 10 min. Appropriate aliquots were injected onto the column.

An alternative extraction was attempted using trichloroacetic acid (TCA). Triplicate 1.0-ml plasma samples containing 6TG or metabolites were precipitated with 1.0 ml 14% TCA (w/v). Following centrifugation at 48,000 *g* for 20 min, 1.0 ml of the supernatant was removed and extracted three times with 1.0 ml diethyl ether. Nitrogen was blown on the aqueous layer for 1.0 min and aliquots injected onto the chromatograph.

A patient (R.E.) who received 1800 mg (1000 mg/m^2) of 6TG as a 60-min intravenous infusion had blood collected into heparinized tubes. The blood samples were centrifuged (700 *g*, 10 min), and the plasma supernatant stored at −20°C until extracted in triplicate with perchloric acid as above.

Quantitation

The areas of 6TG peaks in plasma extracts were compared to a calibration line of peak area versus nmol injected. The calibration line was generated daily by injections from a stock solution of 80 μM 6TG. For extraction efficiency experiments, separate calibration lines were made from stock solutions of each metabolite. These calibration lines were used to calculate relative response factors for the various metabolites compared to 6TG. Subsequently, quantitation of metabolite peaks in patient plasma entailed multiplying the metabolite peak area by the appropriate response factor and comparing this value to a 6TG calibration line. Unknown metabolites are assigned a response factor of 1.0. Quantitation of metabolites detected at 310 nm is carried out in a similar manner with a 6MeTG calibration line.

Analysis of unknowns

Pooled patient urine was adjusted to pH 10 with 10 *N* sodium hydroxide and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate was removed in vacuo and the residue redissolved in 0.01 *M* acetate buffer, pH 3.5. Repeated injections were made onto the analytical column using a mobile phase of 0.01 *M* acetate, pH 3.5—20% methanol which gave baseline resolution of unknowns 1, 2, and 3. After rechromatography, the collected fractions were then alkalinized, extracted into ethyl acetate and dried. The residues were subjected to direct probe electron impact analysis on a VG Micromass 30F (Altrincham, Great Britain) mass spectrometer. The source settings were: 70 eV, 180°C, 4 kV, and 170 μA. UV spectra were obtained on a Cary 118 UV spectrometer (Varian, Palo Alto, CA, U.S.A.). Acidic hydrolysis of unknown 3 was done in 0.1 *N* hydrochloric acid for 10 min at 100°C and analyzed directly by HPLC.

RESULTS AND DISCUSSION

A preliminary separation of 6TG and metabolites was accomplished by a modification (0.01 *M* KH_2PO_4, pH 5.5—10% methanol, isocratic elution) of the method of Hartwick and Brown [10] for nucleosides and bases. However, after less than 100 samples had been injected using this mobile phase for elution, the characteristics of the column suddenly changed as evidenced by a shift in retention times and severe tailing of the 6TG peak. Upon changing the mobile phase to 0.01 *M* sodium acetate, pH 3.4—10% methanol (v/v), the 6TG peak was restored to a sharp and symmetrical shape.

These results prompted a study of the effect of the mobile phase pH and methanol concentration on the resolution of 6TG and related metabolites. Of the compounds studied (6TU, 6TG, 6TX, 6TGS, and 6MeTG), only 6MeTG showed a sensitivity to the pH (Fig. 1). As the pH was raised (with methanol concentration constant at 10%), 6MeTG was eluted at a larger k'. Therefore, when the 6-thio group is methylated, the partition coefficients of these compounds exhibit a sharp dependency on mobile phase pH.

The methanol concentration was varied at pH 3.5 to affect retention. As the polarity of the mobile phase increased with the decreasing methanol concentrations, 6TG and metabolites had longer retention times. An interesting observation is that 6TGS was eluted before 6TX at 15% methanol, but, at lower methanol concentrations it was eluted after 6TX.

Based on these data and the fact that 6MeTG has a λ_{max} at 310 nm and very little absorption at 340 nm, two operating conditions (Fig. 2) were chosen for the study of 6TG pharmacology in patients: (1) elution with 0.01 *M* acetate, pH 3.5—10% methanol and detection at 340 nm for analysis of 6TU, 6TG, 6TX, 6TGS; and (2) elution with 0.01 *M* acetate, pH 3.5—25% methanol and detection at 310 nm for analysis of 6MeTG. Once these conditions were chosen, the mobile phase was premixed and the gradient mixer on the instrument was not used. The premixed mobile phase, however, eluted 6TX and 6TGS together and the k' values for 6TU and 6TG were slightly lowered

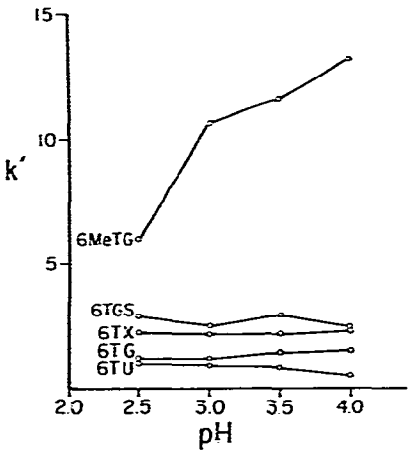

Fig. 1. Capacity factors versus pH of mobile phase. The mobile phase in each case was 0.01 *M* acetate—10% methanol (v/v). 6MeTG was detected at 310 nm.

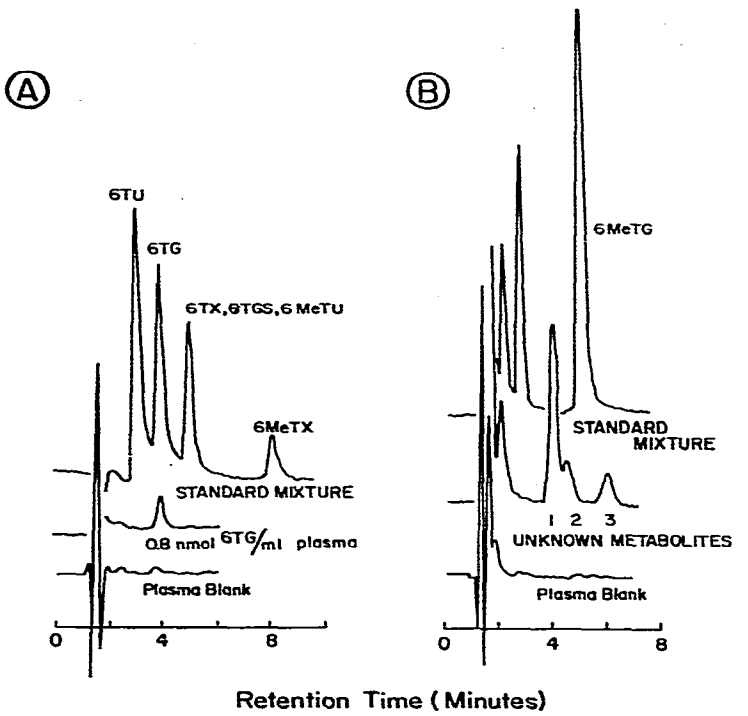

Fig. 2. Chromatograms of 6TG and metabolites in the two isocratic systems used. (A) Elution with 0.01 *M* acetate, pH 3.5—10% methanol and detection at 340 nm. The traces show a plasma blank, the detection limits and a mixture of 6TG and metabolites used as standards. (B) Elution with 0.01 *M* acetate, pH 3.5—25% methanol and detection at 310 nm. The traces show a plasma blank, a typical patient's plasma extract containing the three unknown metabolites, and a mixture of metabolite standards.

TABLE I

COMPOUNDS CHARACTERIZED BY REVERSED-PHASE HPLC

Compound	k'*	Response factor
6TU**	0.57 ± 0.01	1.30
6TG	1.34 ± 0.08	1.00
6TX	1.94 ± 0.08	1.60
6TGS	1.94 ± 0.06	1.78
6MeTU	1.93 ± 0.02	–
6MeTX	3.73 ± 0.05	–
Metabolite 1***	1.72 ± 0.09	1.00
Metabolite 2	2.09 ± 0.14	1.00
6MeTG	2.68 ± 0.02	1.00
Metabolite 3	3.27 ± 0.09	1.00

*Average ± standard deviation of at least four injections using premixed mobile phase.

**Compounds were chromatographed with 0.01 *M* acetate, pH 3.5—10% methanol and detected at 340 nm. Response factors are relative to 6TG.

***Compounds were chromatographed with 0.01 *M* acetate, pH 3.5—25% methanol and detected at 310 nm. Response factors, relative to 6MeTG, are assumed.

(Table I). This observation indicates the probable inaccuracy of the gradient module of the instrument in mixing the methanol concentration and accounts for the differences in k' values between Table I and Figs. 1 and 2. When 6MeTU was obtained and chromatographed, its k' was found to be identical with that of 6TX and 6TGS. Since metabolites seen at this retention time, if present at all, are minor compounds in plasma the resolution of 6TX, 6TGS and 6 MeTU became a moot point, and the premixed 0.01 *M* acetate—10% methanol buffer was kept as the mobile phase in the interest of analysis time.

The extraction efficiencies of 6TG and metabolites with PCA, within the concentration range studied (Table II), gave reasonably consistent values with low standard deviations. Extractions of 6TG in the range of 0.4—3.2 nmol/ml (not shown) gave efficiencies of 38—60% with a lower detection limit of 0.8 nmol/ml (3:1 signal-to-noise ratio). The efficiencies and standard deviations obtained in the concentration range of clinical interest (1—100 nmol/ml) gave confidence in the reproducibility of the assay. An extraction using TCA was also attempted. Although the extraction efficiency was high for 6TG at 32 nmol/ml, the extraction efficiency dropped to low levels for 6TX and 6TU

TABLE II

EXTRACTION EFFICIENCIES OF 6TG AND RELATED COMPOUNDS

Recovery (%), average of 3 extractions ± standard deviation using PCA.

Compound	Concentration (nmol/ml)				
	4	8	16	24	32
6TG	42.5 ± 16	67.0 ± 3.2	82.0 ± 5.2	101.0 ± 8.6	100.3 ± 0.6 (99.7 ± 12.5)*
6TX	76.4 ± 9.8	92.9 ± 10.8	87.4 ± 15.3	101.4 ± 13.3	95.3 ± 5.1 (20 ± 3.5)
6TU	51.9 ± 5.4	48.6 ± 5.3	58.6 ± 1.3	56.4 ± 9.4	69.2 ± 5.7 (32 ± 1.7)
6TGS	63.8 ± 10.4	62.6 ± 2.2	58.2 ± 4.6	58.7 ± 1.6	59.0 ± 2.1
6MeTG	53.0 ± 3.3	78.6 ± 8.1	88.3 ± 4.3	85.2 ± 2.8	95.7 ± 9.2
6TG + guanine**	75.7 ± 3.2	70.9 ± 0.9	84.0 ± 8.5	82.2 ± 1.6	71.1 ± 2.1

*Using TCA extraction.

**1.0 μmol/ml.

(Table II) and this extraction was not investigated further. The use of an internal standard would have been preferred, however, the search for a compound with similar chemical properties and appreciable absorbance near 340 nm was fruitless. All available thiopurines are suspected or proven metabolites except mercaptopurine which coeluted with 6TG.

The pattern of 6TG extraction efficiency indicated that a fixed amount rather than a fixed percent of 6TG was lost in each extract. The extraction study was repeated in the presence of a large excess of guanine (1.0 μmol/ml) in the hope that guanine would saturate any plasma or glassware binding sites for 6TG. The only noticeable improvement in extraction efficiency was at 4 nmol/ml of 6TG where the recovery was increased to 76%. Therefore, guanine was not used as a carrier molecule in subsequent extractions.

The standard plots generated for each metabolite during the extraction efficiency studies were used to compute response factors (Table I) of the compounds relative to 6TG. In all cases the correlation coefficients of the calibration plots were greater than 0.996. These response factors were used to quantify known metabolites with a 6TG calibration line. Response factors were not

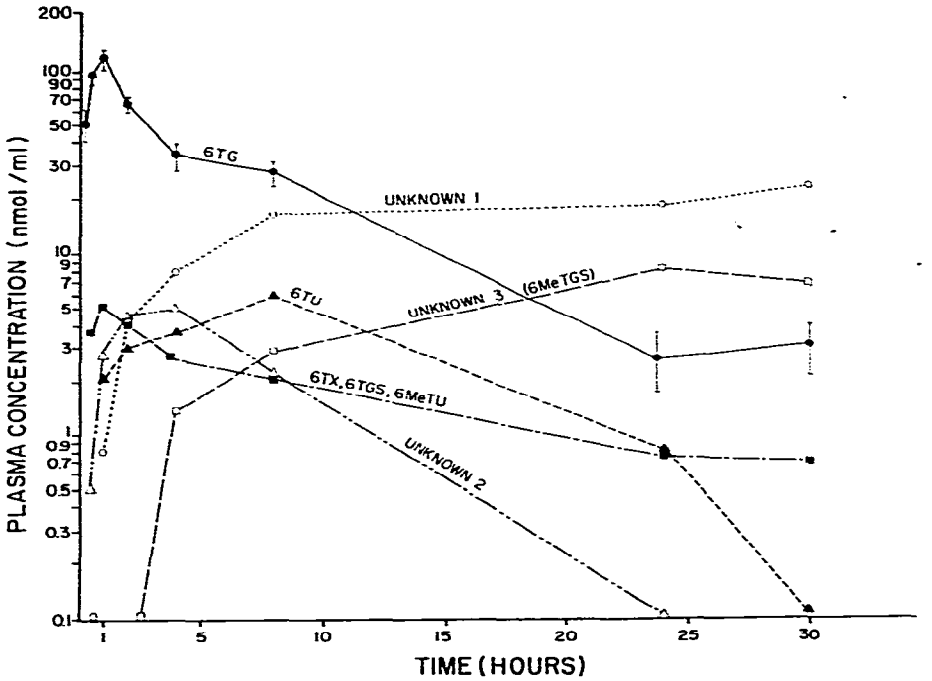

Fig. 3. Plasma concentrations of 6TG and metabolites in patient (L.W.) receiving 1800 mg 6TG as a 60-min infusion. 6TG (●——●), 6TU (▲ - - - ▲), 6TX, 6TGS, 6MeTU (■—— —— ■), metabolite 1 (○ - - - ○), metabolite 2 (△—— - - △), metabolite 3 (□ - - - □). Points represent the mean ± standard deviation of triplicate extractions.

computed for 6MeTX or 6MeTU since there were impurities present in these standards and the concentrations of stock solutions were unknown.

Analysis of extractions from patient plasma revealed, besides large amounts of 6TG, a surprising number of metabolites (Fig. 3). 6TU and 6MeTG (not seen in this patient), were detected as previously reported [2] and identified in extracts by virtue of their retention times. Plasma concentrations of 6TU were detected by 1.0 h and peaked at 8 h. The group of metabolites (6TX, 6TGS, 6MeTU) which are eluted at k' 1.94 peaked at 1 h where they reached a total concentration of 0.045 of the 6TG concentration and declined thereafter. When analyzing the extracts at 310 nm, three peaks, designated metabolites 1, 2, and 3 were seen besides 6MeTG (Fig. 2). The retention time of these peaks did not correspond to any of our known standards. By 24 h, metabolite 1 had become the principal metabolite detected in plasma.

Sufficient quantities of unknown metabolites for structural analysis were obtained by extracting alkalinized pooled patient urine with ethyl acetate. This extraction had an efficiency of greater than 90% for all three unknowns. Attempts at isolating purified unknown metabolites were made by repeated injections onto the analytical HPLC column. Rechromatography of collected fractions, however, revealed that unknowns 1 and 2 degraded rapidly to unknown 3. UV analysis of unknown 3 gave a spectrum similar to 6MeTG (λ_{max} = 310 nm, 1.0 *N* sodium hydroxide; λ_{max} = 328 nm, 1.0 *N* hydrochloric acid). Mild acid treatment caused hydrolysis to 6MeTG as assayed by HPLC. The mass spectrum of unknown 3 (Fig. 4) exhibited the classic fragmentation [11]

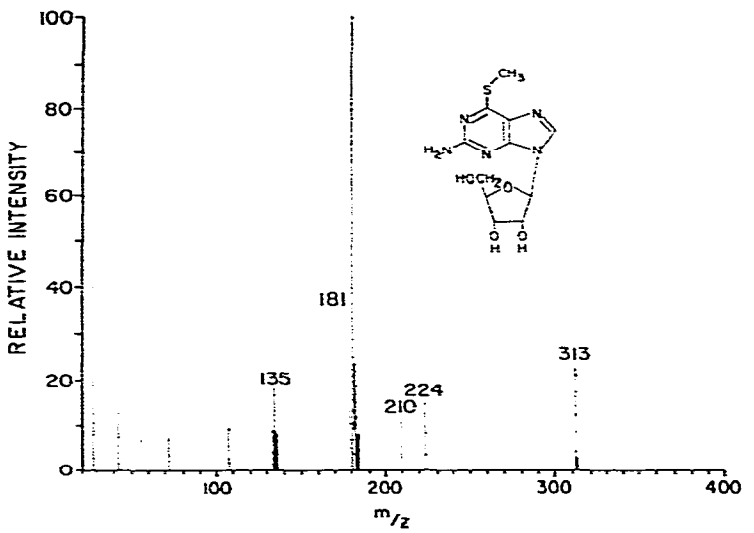

Fig. 4. Mass spectrum of unknown 3. The sample was analyzed by direct probe insertion at 180°C. The metabolite was deduced to be 6-methylthioguanosine.

of a purine riboside [*m/e* 313 $(M)^+$, 224 $(M-89)^+$, 210 $(B+30)^+$, and 181 $(B+H)^+$]. The identity of unknown 3 was concluded to be 6-methylthioguanosine (6 MeTGS). The extractability of unknowns 1 and 2 from base into ethyl acetate, their absorbance at 310 nm, and their high retention on the column suggest that they are 6-thiomethylated metabolites. These characteristics also rule out the possibility of phosphorylated riboside derivatives. All attempts at isolating unknown metabolites 1 and 2 have proven futile and their identity remains undefined.

The finding of 6MeTGS in human plasma is particularly relevant to the recent finding of Lee and Sartorelli [12] that sarcoma 180 cells in vitro actively metabolize 6TG to 6TGS by purine nucleoside phosphorylase. A similar pathway operating in humans, either preceeding or following methylation of the thio functionality, may account for the high concentrations of 6MeTGS we found. Another possibility may be that 6MeTGS is produced from dephosphorylation of 6MeTGS monophosphate during the extraction procedure. To investigate this possibility we extracted guanosine monophosphate (since neither 6MeTGS monophosphate nor 6TGS monophosphate were available) at 20 nmol/ml from plasma and water with PCA as described in Experimental. The extraction efficiency was 97.3 ± 5.5% from water, but dropped to 68.2 ± 2.7% from plasma. The missing monophosphate was totally accounted for by conversion to guanosine. Thus degradation of 6MeTGS monophosphate, if present, may account for some of the 6MeTGS seen in patient plasma; however, considering the high concentrations of 6MeTGS found, a degradative formation would not account for all of the 6MeTGS present.

In either case, the results may question the rationale of using high dose 6TG regimens. High doses of 6TG may merely be shunted into 6TGS and/or 6MeTGS with no correspondingly higher level of the cytostatic 6-thioguanosine monophosphate attained.

In summary we have delineated an isocratic reversed-phase HPLC method for

the routine analysis of 6TG and metabolites in human plasma. We have observed three previously undescribed metabolites in humans and we have definitively identified one of them as 6-methylthioguanosine.

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