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Reversed-phase high-performance liquid chromatographic approach to determine total lymphocyte concentrations of 6-thioguanine, methylmercaptopurine and methylthioguanine in humans

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) procedure was developed to quantify intracellular lymphocyte 6-thioguanine, methylmercaptopurine and methylthioguanine. The free base of each metabolite was obtained by acid hydrolysis, which allowed for a total determination of thiopurine metabolites. 6-Thioguanine was analyzed on an octadecylsilane column using acetonitrile–10 m*M* sodium phosphate (11:89), pH 7, containing 0.06% tetrabutylammonium chloride. 6-Thioguanine was oxidized with potassium permanganate, and fluorescence was measured at 330 nm excitation and 410 nm emission. Methylmercaptopurine and methylthioguanine were separated on a cyanopropylsilane column using methanol–40 m*M* sodium phosphate (22:78), pH 2.7, and detected by ultraviolet absorbance at 314 and 290 nm, respectively.

INTRODUCTION

The use of thiopurines to treat acute lymphoblastic leukemia (ALL) has resulted in 90% remission; however, 30–40% of children relapse while receiving maintenance therapy. Consequently, these patients are more likely to die of their disease. ALL patients, as well as those who undergo thiopurine therapy for other reasons such as organ transplantation and Crohn's disease, are at a constant risk

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of developing leukemia secondary to thiopurine therapy. Dosing these patients empirically by body weight and clinical outcome has not resulted in uniformly safe and effective drug therapy for every patient.

The target cell of 6-mercaptopurine (MP) therapy is the lymphocyte. In order to induce and maintain remission of ALL, the neoplastic lymphoblasts must be eliminated. To control other diseases that require suppression of the immune system, it is also the lymphocyte that must be regulated. The primary problem associated with studying lymphocytes is the need for a large number of cells to compensate for poor assay sensitivity. Previous methods have relied on erythrocyte (red blood cell, RBC) samples principly for that reason.

Early investigations to quantify thioguanine (TG) nucleotides *in vitro* concentrated primarily on L5178Y murine lymphoma tissue cultures [1–4]. Analyses used to quantify thiopurine metabolites in cell culture included paper chromatography [1], radiolabeled assays [2,3], and anion-exchange chromatography with fluorescence spectroscopy [4].

The current method for evaluating thiopurine metabolites as clinical markers of myelosuppression has not changed significantly. Lennard and Maddocks [5] have studied the TG nucleotide concentrations of RBCs by isolating the nucleotides as lanthanum salts, hydrolyzing the molecule to free the purine base, and quantifying the fluorescent sulfonate derivative. A more specific method developed by Lavi and Holcenberg [6] quantified the individual mono-, di- and trinucleotides of thioinosine and TG in RBCs. The procedure isolated thiopurines by adsorption to a mercurial cellulose resin followed by anion-exchange chromatography.

Fletcher and Maddocks [7] developed a method to quantify 6-thioinosinic acid in human lymphocytes *in vivo*. The procedure hydrolyzed the extracted nucleotides and quantified the free MP base. A similar procedure was used by Dooley and Maddocks [8], but the thioinosinic acid was extracted from RBCs and analyzed as the nucleotide using anion-exchange chromatography.

Weinshilboum *et al.* [9] identified an enzyme, thiopurine methyltransferase (TPMT), that S-methylates thioinosinic acid and TG nucleotide to form methylthioinosinic acid (MTIA) and methylthioguanylic acid (MTG), respectively. Erdmann *et al.* [10] developed a high-performance liquid chromatographic (HPLC) method for determining the total concentration of MTG and methylmercaptopurine (MMP), as well as TG and MP, in RBCs of patients on thiopurine therapy. The study showed a significant accumulation of MMP and lesser amounts of MTG in RBCs.

In an attempt to identify the relationship between MP therapy and toxicity, we developed an HPLC method capable of detecting low nanogram quantities of TG, MMP and MTG in lymphocytes. The lymphocyte method is very sensitive and requires only 10 ml of whole blood. The assay procedure is similar to the RBC method by Erdmann *et al.* [10] that hydrolyzes the nucleotides and nucleosides to free bases before analysis. The lymphocyte sample preparation is cleaner

than working with RBCs which has allowed for improved sensitivity. An application of this method to a patient with Crohn's disease is also presented.

EXPERIMENTAL

Materials

All chemicals were analytical grade except where noted. The TG, MMP, MTG, sulfamethoxazole (SMX), ethyleneglycol tetraacetic acid, Ficoll-Hypaque and hydrogen peroxide were obained from Sigma (St. Louis, MO, USA). Sulfuric acid, methanol (HPLC grade) and water (HPLC grade) were purchased from Mallinckrodt, (Paris, KY, USA). Perchloric acid (70–72%) sodium bicarbonate, sodium dihydrogenphosphate, potassium permanganate, sodium hydroxide, hydrochloric acid, dichloromethane and ethyl acetate were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Tetrabutylammonium chloride (HPLC grade) was obtained from Eastman Kodak (Rochester, NY, USA).

Instrumentation

The chromatography was performed with a Hewlett-Packard Model 1090 liquid chromatograph equipped with a data processing unit, diode-array detector, heated column compartment, auto-sampler and auto-injector, and a model 1046 fluorescence detector with a xenon lamp (Hewlett-Packard, Palo Alto, CA, USA). Fluorescence peak heights were determined with a Hewlett-Packard Model 3392 integrator. Cell counts were obtained with a Coulter counter, Model Zf (Coulter Electronics, Hialeh, FL, USA).

Chromatographic conditions

(a) Thioguanine was separated by pumping a mobile phase consisting of acetonitrile–10 m*M* sodium phosphate (11:89, v/v), through a 200 mm × 4.6 mm octadecylsilane column (Hypersil ODS, 5 μm, Hewlett-Packard). The buffer contained 0.06% (w/v) tetrabutylammonium chloride and was adjusted to pH 7. The flow-rate was 1.5 ml/min. Fluorescence of TG was monitored at 330 nm (12 nm bandwidth) excitation and 410 nm (25 nm bandwidth) emission, with a photomultiplier setting of 15. A 370-nm cut-off filter was used to block any light from 370 nm and lower from entering the photomultiplier. The column compartment was heated to 45°C, and 10 μl were injected onto the column.

(b) MMP and MTG were separated on a 150 mm × 4.6 mm cyanopropylsilane column (Zorbax CN, 5 μm, Dupont, Biomedical Products, Wilmington, DE, USA). A mobile phase consisting of methanol–40 m*M* sodium phosphate (22:78, v/v) was pumped at 1.5 ml/min through the column. The buffer was adjusted to pH 2.7 before mixing with the methanol. The metabolites were detected by simultaneously monitoring UV absorbance at 290 nm (4 nm bandwidth) for MTG and 314 nm (4 nm bandwidth) for MMP.

Drug solutions

A 20 μg/ml stock solution of TG was prepared by first dissolving the drug in 0.1 *M* NaOH, then bringing to final volume with 0.1 *M* HCl. A stock solution containing 120 μg/ml MMP and 300 μg/ml MTG was prepared in methanol. All stock solutions were diluted 1:10 and stored at 4°C until the standard curves were prepared. The stock solutions were prepared fresh daily. SMX, the internal standard (I.S.) for the MMP–MTG assay, was dissolved in methanol at 200 μg/ml.

MMP–MTG extraction solution

The extraction solution was prepared using ethyl acetate–dichloromethane (25:75, v/v) containing 0.1 μg/ml SMX.

Sample preparation

For purposes of quantification, all drug concentrations were normalized to $5 \cdot 10^6$ cells. All standard curves were constructed with $n = 4$ replicates for each concentration point. Aqueous dilutions of TG, MMF and MTG were prepared and 100 μl of each dilution were added to tubes containing 100 μl of washed lymphocytes. The standard curve concentration range was 1, 2, 4, 10, 15, 30 and 40 ng for TG, 2, 6, 12, 20 and 40 ng for MMP, and 5, 15, 30, 40 and 50 ng for MTG per 200 μl. Then, either 200 μl of aqueous standard solution or 200 μl of patient sample was added to 50 μl of 1 *M* sulfuric acid and heated for 45 min at 100°C. The hydrolysate was cooled and centrifuged, and 200 μl were placed into a clean tube containing 55 μl of 1 *M* sodium bicarbonate, pH 10.1. The samples were extracted with 1.5 ml of extraction solution, vortex-mixed and centrifuged. A 175-μl aliquot of the upper aqueous layer was transferred to a clean tube containing 25 μl of 1 *M* sodium bicarbonate, pH 10.1. TG was analyzed in the aqueous fraction by oxidizing the drug with 25 μl of 0.5% potassium permanganate for 5 min, then stopping the reaction with 5 μl of 15% hydrogen peroxide. The samples were vortex-mixed and transferred to HPLC vials. The remaining organic fraction was analyzed for MMP–MTG by evaporating the solvent to dryness in a 40°C water bath under a stream of nitrogen. The dried residue was reconstituted in 100 μl of methanol.

Quantification

Due to the limited availability of standards for thiopurines, a suitable I.S. for TG was not found. Drug levels were determined according to the peak height of external standards. An I.S. was used for MMP–MTG quantitation. The peak-height ratios of MMP and MTG to I.S were plotted against concentration.

Patient sample

A 10-ml blood sample was collected from a patient diagnosed with Crohn's disease receiving MP for immunosuppressive therapy. The sample was diluted with 15 ml of phosphate-buffered saline (PBS) containing 3 m*M* ethyleneglycol

tetraacetic acid. The lymphocytes were isolated by layering 8 ml of diluted blood on 2 ml Ficoll-Hypaque and centrifuging at 9000 *g* for 20 min. The lymphocyte band was collected and washed with PBS, and the cells were resuspended in 250 μl of PBS. Cell density was determined with a Coulter counter, and the remainder of the sample was stored at −70°C until analyzed.

Absolute recovery

Lymphocyte samples containing known concentrations of MMP (2, 12, 40 ng), MTG (5, 30, 50 ng) and TG (1, 4, 15 ng) were compared with corresponding concentrations of drug prepared in water to simulate 100% recovery. The two standard curves were analyzed together. The recovery was determined by calculating the drug concentrations found in lymphocytes using the linear regression equation obtained from the 100% recovery curve, dividing the lymphocyte concentration by the recovery concentration and recording the difference as a percentage.

Statistics

Linearity was calculated by least-squares regression analysis and reported as r^2. The intercept and slope estimates included standard deviations. The values representing the intercept and slope for TG are based on arbitrary peak-height units assigned by the integrator. All standard deviation measurements used in calculating between-day and within-day precision were sample standard deviations ($n - 1$).

RESULTS

Linearity and precision

The observed linearity and precision for TG, MMP and MTG was good over the concentration ranges studied. Between-day precision was determined by preparing the high and low values for each standard curve and analyzing them in quadruplicate over three days.

TG. The r^2 for TG was 0.9944 with a slope of 2897.2 ± 29.5 and an intercept of −195.2 ± 1754.8. Within-day precision ranged from 11.7% at 1 ng, 2.4% at 20 ng and 3.4% at 40 ng. Between-day precision ranged from 13.2% at 1 ng, 3.7% at 15 ng and 2.5% at 40 ng.

MMP and MTG. The r^2 for MMP was 0.9934 with a slope of 0.0240 ± 0.0005 and an intercept of 0.0254 ± 0.0277. The r^2 for MTG was 0.9781 with a slope of 0.0141 ± 0.0005 and an intercept of 0.0219 ± 0.0363. Within-day precision for MMP ranged from 23.4% at 2 ng, 5.7% at 12 ng and 4.6% at 40 ng; for MTG, 11.4% at 5 ng, 6.8% at 30 ng and 4.2% a 50 ng. Between-day precision for MMP was 19.1% at 2 ng, 3.9% at 20 ng and 4.1% at 40 ng; for MTG, 18% at 5 ng, 8.1% at 30 ng and 3.7% at 50 ng.

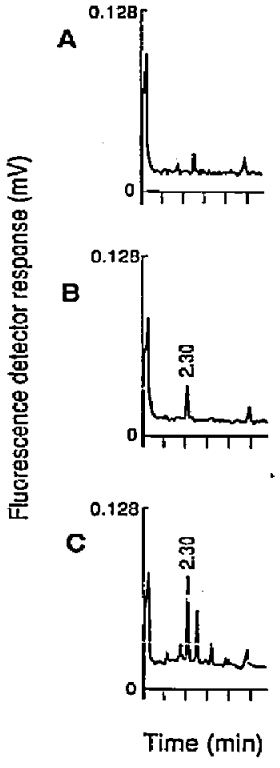
Absolute recovery

The mean recovery of MMP was 37%, MTG was 38% and TG was 81%. The extraction efficiency of MMP under these conditions was similar to other reports [4], however, MTG recovery could be increased to 60% by using ethyl acetate alone. TG remains in the aqueous fraction which migrates to the top of the tube when using ethyl acetate–dichloromethane. We have found it easier to first remove the aqueous layer at the top of the tube, and then the organic at the bottom of the tube. If only MMP and MTG were to be analyzed, ethyl acetate alone would be the extraction solvent of choice.

Lymphocyte analysis

Chromatograms showing TG and the resolution of MMP–MTG are shown in Figs. 1 and 2. There were no interfering substances present in blank lymphocytes (Figs. 1A and 2A). A chromatogram of a TG standard (3 ng) is shown in Fig. 1B. TG eluted at 2.30 min. A Crohn's patient sample (Fig. 1C) showed 8 ng TG after normalizing to $5 \cdot 10^6$ lymphocytes.

Chromatograms representing the analysis of MMP and MTG are shown in Fig. 2. Fig. 2B shows a standard containing 6 ng MMP and 15 ng MTG. MMP



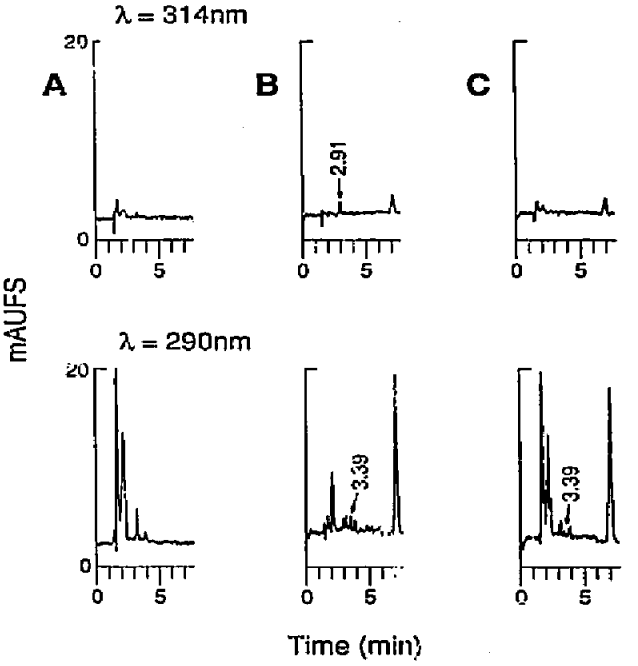


Fig. 1. Chromatograms of TG in lymphocytes. (A) Blank lymphocytes; (B) 3 ng TG per $5 \cdot 10^6$ lymphocytes standards; (C) Crohn's patient sample showing 8 ng TG per $5 \cdot 10^6$ lymphocytes. TG = 2.30 min.

Fig. 2. Chromatograms of MTG (314 nm) and MMP (290 nm) in lymphocytes. (A) Blank lymphocytes; (B) 15 ng MTG and 6 ng MMP per $5 \cdot 10^6$ lymphocytes standard; (C) Crohn's patient sample showing 1.1 ng MMP per $5 \cdot 10^6$ lymphocytes. MTG = 2.91 min; MMP = 3.39 min.

eluted at 3.39 min, MTG at 2.91 min and the I.S. at 7.15 min. The Crohn's patient sample (Fig. 2C) showed an MMP concentration of 1.1 ng after normalizing to $5 \cdot 10^6$ lymphocytes. No MTG was detected.

DISCUSSION

MP and azathioprine are primary agents in treating patients with ALL, organ transplants and certain autoimmune disorders (*e.g.* Crohn's disease). The incidence of delayed cytotoxicity and life-threatening myelosuppression and infection, resulting from toxic metabolite formation, is high and may only be identified through clinical observation.

In an effort to determine the significance of methylated metabolites *in vivo*, Erdmann *et al.* [10] studied the total concentrations of MTG and MMP, as well as TG and MP, in RBCs of patients on thiopurine therapy. This procedure quantified the total thiopurine content utilizing acid hydrolysis of the nucleotides in place of enzyme hydrolysis. Quantitation of the free thiopurine bases was then performed using commercially available standards. Results of the report suggested that significant levels of intracellular RBC MMP accumulate in these patients with lesser amounts of MTG being present.

Lymphocytes are the target cells in thiopurine therapy. The ability to quantify thiopurine metabolites in lymphocytes has been limited by assay sensitivity and, unlike studies with RBCs, the availability of sufficient cell numbers for *in vivo* studies. However, based on our current HPLC methodology, TG sensitivity has been validated to 1 ng and detection may be enhanced to 200–300 pg by reducing the column length and increasing the injection volume to 15 μl. It is also possible that the use of a mercurial cellulose resin to concentrate the thiopurine metabolites, such as that described by Lavi and Holcenberg [6], could increase the sensitivity of the assay, or possibly improve the precision of the smallest concentrations. However, the ability of the methylated metabolites to bind to mercurial derivatives has not been fully studied. The oxidation of thioguanine to the sulfonate increases detectability significantly, however, the methylated metabolites cannot be oxidized and must be detected by less sensitive UV absorption.

The lack of thiopurine nucleotide standards has further complicated the analysis of these metabolites *in vivo*. Researchers have obtained nucleotides by isolating and purifying minute quantities of nucleotides from cell cultures. In other reports peak shift analyses have been employed by collecting and treating "suspect" chromatographic peaks with specific enzymes, and following the resultant shift of the peak during subsequent chromatographic runs using commercially available standards [2,3].

The reversed-phase HPLC method described here is sufficiently sensitive to quantify total intracellular pools of TG, MMP and MTG in lymphocytes obtained from a 10-ml whole blood sample. We are currently using this method to determine the effect intracellular thiopurines have on cellular adenine and guanine levels.

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