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Journal of Chromatography B, 757 (2001) 1–9

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Improved methods for determining the concentration of 6-thioguanine nucleotides and 6-methylmercaptopurine nucleotides in blood

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Received 13 December 1999; received in revised form 7 September 2000; accepted 14 September 2000

Abstract

The conversion of the cytotoxic and immunosuppressive 6-mercaptopurine (6MP) to the active 6-thioguanine nucleotides (6TGN) is necessary for clinical efficacy of 6MP and its prodrug azathioprine. Another metabolite, 6-methylmercaptopurine nucleotide (6MMPN), is formed via a competing pathway by thiopurine methyl transferase. The concentrations of 6TGN and 6MMPN are measured in washed erythrocytes as a surrogate to the intracellular levels of these metabolites in the target tissues. Analysis of 6TGN and 6MMPN in multi-center clinical studies is more complicated because of the requirement to wash erythrocytes. In this investigation, we found no differences in the concentrations of 6TGN and 6MMPN in blood versus washed erythrocytes in samples obtained from patients taking therapeutic doses of oral 6MP or azathioprine for inflammatory bowel disease. We concluded that whole blood could be used for the analysis of these analytes, thus saving sample preparation time. We also found that the erythrocyte 6TGN concentration in blood at ambient temperature declined 2–4% per day, a loss that can be avoided by shipping blood samples frozen. The loss of 6TGN in blood stored at approximately −80°C was 1% after 1 week and 12% after 24 weeks, indicating the analyte was moderately stable. 6MMPN in blood did not significantly change after 24 weeks of storage at approximately −80°C. In addition, the sensitivity of the 6TGN assay was improved by modifying the HPLC conditions, which made the method more suitable for quantifying low levels of 6TGN in human intestinal biopsy samples and blood.

Keywords: 6-Thioguanine nucleotides; 6-Methylmercaptopurine nucleotides

1. Introduction

6-Mercaptopurine (6MP), and its prodrug azathioprine (AZA), are cytotoxic and immunosuppressive drugs used to treat numerous conditions, including childhood acute lymphoblastic leukemia (ALL), organ transplant rejection, and inflammatory bowel disease (IBD). Metabolism of 6MP is necessary for clinical efficacy [1]. The conversion of 6MP to 6-thioinosine 5′-monophosphate, which is catalyzed by hypoxanthine phosphoribosyltransferase (HPRT) [1], is the first step in formation of the

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00513-2

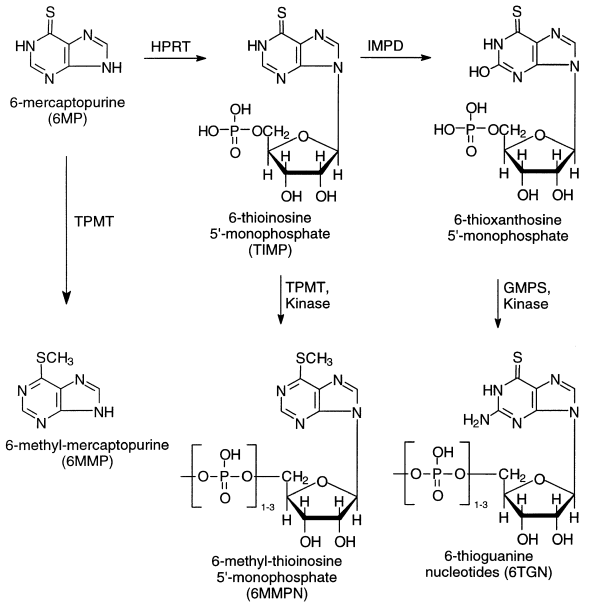

Fig. 1. Competing pathways of metabolism of 6MP to 6TGN and 6MMPN [1,17]. HPRT, hypoxanthine phosphoribosyltransferase; IMPD, inosine monophosphate dehydrogenase; TPMT, thiopurine methyltransferase; GMPS, guanosine monophosphate synthetase.

active metabolite, 6-thioguanine nucleotides (6TGN, Fig. 1). The metabolites 6-methylmercaptopurine (6MMP) and 6-thiouric acid are formed by thiopurine methyl transferase (TPMT) [2,3] and xanthine oxidase, respectively [1,4]. 6MMP nucleotide (6MMPN) is formed by the methylation of 6-thioinosine 5′-monophosphate by TPMT [5].

Variation in the clinical response to 6MP has been linked to the genetic polymorphism of TPMT [3,6]. An inverse relationship exists between TPMT activity and the concentration of 6TGN in red blood cells (RBC) [6]. Accurate dosing of 6MP is imperative for clinical efficacy [7,8]. 6TGN and 6MMPN are assayed by first washing erythrocytes followed by acidic hydrolysis of intracellular nucleotides. The liberated thiopurine bases are extracted and quantified by one of several methods that use HPLC with UV or fluorescence detection [9–12]. We chose to use the assays of Erdmann and coworkers in which the 6-thioguanine (6TG) that was recovered from hydrolysis of the nucleotides was oxidized to the corresponding sulfonate before quantifying by fluorescence [12]. The hydrolysis product of 6MMPN was quantified by UV absorbance. These assays measure the total concentrations of 6TG or 6MMP thiopurine base and their corresponding nucleosides and nucleotides (mono-, di-, and triphosphates) in the RBC [12,13]. The metabolism of purines includes a cycling between thiopurine base, mono-, di-, and triphosphates due to, in part, the purine salvage pathways [14,15]. The intracellular concentration of each of these forms present is time dependent [10]. Therefore, the total 6TGN and 6MMPN concentrations are quantified to measure the total cytotoxic burden to cells [10,12].

Previous to our investigation the concentrations of 6TGN and 6MMPN in RBC have been quantified in washed erythrocytes. This ensured that the 6TGN and 6MMPN in other components of blood would not contribute to that measured for RBC. However, the concentrations of these analytes in the neutrophils and lymphocytes are too low to contribute significantly to that measured for RBC [16]. The concentrations of 6TG and 6MMP in the plasma of patients with IBD who are treated with low, oral doses of 6MP have not been measured, but are probably low based on levels reported for patients receiving high intravenous doses of 6MP [17,18]. Using blood would allow samples to be frozen immediately after collection, thus minimizing further analyte metabolism and decomposition that occurs during shipment of blood at ambient temperature. Omission of the washing of RBC also would decrease the time of sample preparation.

In this investigation, we report improvements for the assays of 6TGN and 6MMPN in blood. Data are presented that support our hypothesis that 6TGN and 6MMPN concentrations in blood and washed erythrocytes are the same. We present for the first time data describing the stability of 6TGN and 6MMPN in frozen blood. Finally, we apply the improved assay for 6TG in blood to measure 6TG in the intestinal tissue of an IBD patient receiving 6MP therapy.

2. Experimental

2.1. Reagents

6TG, 6MMP, sodium phosphate monobasic and sodium phosphate dibasic were purchased from

Sigma Chemical Company, St. Louis, MO. HPLC grade ethyl acetate, dichloromethane, 70% (w/v) perchloric acid, and 30% (w/v) hydrogen peroxide were obtained from Fisher Scientific (Fair Lawn, NJ). Aldrich Chemical Company (Milwaukee, WI) supplied potassium permanganate, tetrabutylammonium chloride, and sulfamethoxazole. J.T. Baker (Phillipsburg, NJ) supplied hydrochloric acid and sulfuric acid. HPLC grade acetonitrile was purchased from EM Science (Gibbstown, NJ) and Curtin Matheson Scientific (Houston, TX) supplied the sodium bicarbonate. The 6-thioguanine sulfonate was synthesized and purified as described previously [19], and was >97% pure by HPLC–UV. Patient blood was collected in either Vacutainer (Becton Dickinson, Franklin Lakes, NJ) or Monoject (Sherwood Medical, St. Louis, MO) tubes containing K_3 EDTA.

2.2. Instrumentation and HPLC conditions

The HPLC consisted of a Shimadzu (Kyoto, Japan) SCL-10A system controller and LC-10AD pump, FCV-10AL mobile phase mixer, GT-104 mobile phase degasser, and an SIL-10A auto injector. A Shimadzu RF-10A spectrofluorometric detector and a Linear 204 UV/VIS detector were used for the 6TGN and 6MMPN analyses, respectively. Data were collected and analyzed with Class-VP software, version 4.2.

The 6TG sulfonate was separated on a Hewlett–Packard (Wilmington, DE) ODS Hypersil column (200×4.6 mm, 5 μm) at 45°C using an Eppendorf (Brinkman, NY) CH-30 column heater. The mobile phase of 85% aqueous (10 m*M* phosphate buffer, 2 m*M* tetrabutylammonium chloride, pH 7.0) and 15% acetonitrile (v/v) was run at 1.2 ml/min. The aqueous portion of the mobile phase was made by the addition of 7.8 ml 1 *M* monobasic and 12.2 ml 1 *M* dibasic sodium phosphate to 2 l water. Hydrochloric acid (1:1, v/v) was used to bring the pH to 7.0. To this 45 ml acetonitrile and 1.446 g tetrabutyl ammonium chloride was added. The analyte was quantified by fluorescence detection (excitation λ= 330 nm, emission λ=410 nm).

6MMP was separated from interfering substances and the internal standard, sulfamethoxazole, on a Phenomenex (Torrance, CA) Hypersil C8 BDS column (150×4.6 mm, 5 μm) fitted with the accompanying guard column (30×4.6 mm). UV absorbance was monitored at 288 nm. The mobile phase, containing 85% 10 m*M* sodium phosphate (9.35 ml 1 *M* monobasic and 0.65 ml 1 *M* dibasic in 1 l, pH to 5.75 with concentrated NaOH or HCl) and 15% acetonitrile (v/v), was run at a flow-rate of 1.2 ml/min.

2.3. Sample preparation-6TGN

Standards and samples were assayed as described previously [12] with the following modifications. The 6TG stock solution (80 μg/ml) was prepared by dissolving 6TG in 5 ml 0.1 *M* NaOH and diluting to 50 ml with water. This solution was stored at 4°C in the dark and was stable for at least one month by HPLC–UV (data not shown). Whole blood or washed RBC (100 μl), water (150 μl), 6TG standard (100 μl), and 1 *M* sulfuric acid (200 μl) were heated in 1.5 ml polypropylene screw cap tubes (Sarstedt, Germany) for 45 min at 100°C. The samples were centrifuged at 3500 *g* for 10 min and the supernatant (250 μl) was transferred to a new tube for precipitation with 23% (w/v) perchloric acid (10 μl). This solution was extracted with 1 ml dichloromethane and centrifuged for 10 min at 13 000 *g*. Aqueous supernatant (125 μl) was transferred to a clean 12×75 mm glass tube and 1 *M* sodium bicarbonate, pH 10.1 (150 μl) was added to adjust the solution to approximately pH 9. Potassium permanganate (32 m*M*, 25 μl) was added to oxidize 6TG to its fluorescent sulfonate [19]. After 5 min, this reaction was stopped with 15% hydrogen peroxide (5 μl). The sample was centrifuged for 10 min at 13 000 *g*, and 5 μl of the supernatant were injected on the HPLC. The stability of the 6TG sulfonate on the autosampler was determined by injecting three concentrations on the HPLC in triplicate over time. Under these conditions, the 6TG sulfonate was stable on the autosampler for 16 h (data not shown).

Standards (2.5, 5, 10, 20, 30, 40, and 50 ng/100 μl) were prepared by spiking 6TG into blood or washed RBC. Standard curves were constructed using peak areas and analyzed by linear regression with $1/y$-weighting. The lower limit of quantification used (2.5 ng/100 μl) was equivalent to approximately 30 pmol/8×10^8 RBC.

2.4. Sample preparation-6MMPN

Samples were prepared for 6MMPN analyses as described previously [12] with minor modifications. 6MMP stock solution (80 μg/ml) was prepared in methanol and was stable for at least one year by HPLC/UV stored at 4°C (data not shown). Briefly, washed RBC or whole blood (100 μl), standard 6MMP (100 μl), and 0.5 *M* sulfuric acid (200 μl) were mixed and heated in 1.5 ml polypropylene screw-cap tubes (Sarstedt, Germany) at 100°C for 40 min. The samples were centrifuged for 10 min at 3500 *g* and the supernatant (250 μl) was added to 0.5 *M* sodium bicarbonate, pH 10.1 (125 μl). The 6MMP was extracted into ethyl acetate (2 ml) containing sulfamethoxazole (1.5 μg/ml) as the internal standard for volume control. The organic layer was separated by centrifugation (15 min, 2200 *g*), transferred to a clean tube, and evaporated to dryness at 50°C under nitrogen in a model 112 N-Evap (Organomation Association Incorporated, Berlin, MA). The residue was reconstituted in mobile phase (100 μl) and 45 μl were injected on the HPLC. The 6MMP in reconstituted samples was stable for 24 h on the autosampler at room temperature (data not shown).

Standards (20, 50, 100, 200, 500, 1000, 2000, and 3000 ng/100 μl) were prepared by spiking 6MMP into either blood or washed RBC. Standard curves were constructed from the peak area ratio of the analyte to internal standard. The data were fit by linear regression analysis with $1/x$-weighting and provided a lower limit of quantitation of 20 ng/100 μl and a limit of detection of 10 ng/100 μl ($S/N=2$). There was no signal greater than noise at the retention time for 6MMP in chromatograms from six blood donors not receiving 6MP.

2.5. Degradation of 6TGN in blood at ambient temperature

Blood (ten 5-ml samples from each patient) was collected from three IBD patients receiving 100 or 150 mg AZA/day (2 patients) or 75 mg 6MP/day (one patient) prior to their morning dose. The blood samples were maintained at room temperature throughout the study. On each day for seven days, the RBC from one 5-ml sample were washed as described in Table 1. A sample was taken for a RBC count and 1-ml aliquots were stored at approximately −80°C. Three weeks later, samples were thawed and assayed on the same day for 6TGN as described above.

2.6. Improved 6TGN assay

After completion of the above studies, we modified the assay for 6TGN in blood and applied this improved assay to determine 6TGN concentrations in human duodenum. Using the standard chromatography conditions above, a peak eluted at the same retention time as our peak of interest, the 6TG sulfonate, in blank samples. The size of the interfering peak increased over time, thereby limiting the number of samples we could process per batch and the sensitivity of the assay. To eliminate these problems, the organic modifier was reduced to 5% acetonitrile and the flow-rate increased to 1.7 ml/min. In addition, blood (100 μl), 6TG standard (20 μl), water (100 μl), and 3 *M* sulfuric acid (25 μl) were used for the 100°C hydrolysis. After cooling to 4°C, perchloric acid and dichloromethane were added directly to the mixture. Following centrifugation at 13 000 *g* for 10 min, the aqueous supernatant (125 μl) was transferred to a clean tube and oxidized as above. The limits of quantification and detection for the assay were 2.5 ng 6TGN/100 μl blood and 0.5 ng/100 μl ($S/N=2$), respectively. There was no signal greater than noise at the retention time for 6TG in chromatograms from six blood donors not receiving 6MP.

To determine 6TGN in intestinal tissue, approximately 20 mg of frozen duodenum (4–5 biopsies) were weighed in a tared 12×75 mm glass tube. An appropriate volume of 32 m*M* dithiothreitol (DTT) was added to achieve a concentration of 20 mg tissue/ml. The sample was kept on ice until it was homogenized with a Polytron homogenizer (Brinkman, NY) for 2 min at 6000 rpm. The homogenate (200 μl), 6TG standard (20 μl), and 3 *M* sulfuric acid (25 μl) were used for the hydrolysis step, and the assay was continued as described above in the improved 6TGN assay for blood. The standard curve for the tissue analysis was constructed from blank duodenal homogenate spiked with 0.37–15 pmol 6TGN/mg tissue.

Table 1
6TGN and 6MMPN analyzed in blood and washed RBC[a]

Patient	6TGN (pmol/8×10^8 RBC)			6MMPN (pmol/8×10^8 RBC)		
	Washed RBC	Whole blood	Percent of washed RBC	Washed RBC	Whole blood	Percent of washed RBC
1	223±25	280±8	126	3416±728	3606±516	106
2	107±7	119±5	111	767±37	837±35	109
3	110±3	123±2	112	962±191	1077±79	112
4	142±22	153±13	108	390±47	354±29	91
5	649±115	609±24	94	<LOQ	<LOQ	
6	212±8	223±35	105	15 622±1494	13 991±1281	90
7	227±13	214±13	94	5532±814	4738±123	86
8	359±30	329±17	92	498±37	449±25	90
Mean±90% CI			105±20			98±19

[a] Blood samples (10 ml) were collected from 8 donors with either Crohn's disease (n=7) or ulcerative colitis (n=1) receiving AZA (4 patients, 150–250 mg/day) or 6MP (4 patients, 75–125 mg/day) to compare metabolite levels in washed RBC and blood. The blood sample was mixed, a sample was removed for a RBC count, and 5 ml was frozen as whole blood. The remaining blood was centrifuged at 1300× g for 10 min, the plasma and buffy coat were removed, and the RBC were washed twice with 2 volumes of phosphate buffered saline (PBS). The RBC were resuspended in an equal volume of PBS, a sample was removed for an RBC count, and the remainder was stored in 1 ml aliquots at approximately −80°C. Samples were analyzed as described in the Experimental section. Values are the mean±90% CI, n=3. Values less than the limit of quantitation for the assay are designated by <LOQ.

3. Results and discussion

Clinical outcome for patients receiving 6MP or AZA therapy varies widely due to, in part, the common genetic polymorphisms of TPMT [3,6]. The utility of quantifying metabolites of 6MP in washed RBC for predicting the therapeutic and toxic effects of 6MP has been the focus of numerous investigations [16,20,21]. However, there are disadvantages to using washed erythrocytes. The sample of blood must be shipped unfrozen to the analytical laboratory. This can result in a significant delay (typically 2–5 days for multi-center trials) between collecting and freezing of the sample, during which time the analytes can degrade. In blood samples from three IBD patients receiving 6MP, the 6TGN levels in erythrocytes declined at 1.7±0.5, 3.2±0.7, and 4.3±0.6 percent per day over six days at ambient temperature (slope±SE, n=29–34). After the sample is received by the laboratory, a time consuming series of centrifugation steps is necessary to separate and wash the RBC.

In this study we hypothesized the RBC 6TGN and 6MMPN levels measured in the whole blood of IBD patients would not be different from those measured in washed RBC. Blood samples from eight patients receiving either AZA (150–250 mg/day) or 6MP (75–125 mg/day) were collected and frozen as both blood and washed RBC. The measured 6TGN and 6MMPN levels are shown in Table 1. Levels for the eight subjects ranged from 649±115 to 107±7 pmol/8×10^8 RBC for 6TGN. Concentrations of 6MMPN in the RBC ranged from 15 622±1494 pmol/8×10^8 RBC to less than our limit of quantification (240 pmol/8×10^8 RBC). With 8 subjects, α=0.05, the 6TGN study has a power of 0.86 to detect a 15% difference between the two methods of sample preparation using a paired t-test. A paired t-test indicated no difference (P=0.81) between the concentration of 6TGN in blood and 6TGN in washed RBC when normalized to 8×10^8 RBC. A paired t-test could not be used for the 6MMPN data because the data were not distributed normally. The concentration of 6MMPN in blood was the same as that in washed RBC when normalized to 8×10^8 RBC according to the Wilcoxon signed rank test (P=0.81).

These results show 6TGN and 6MMPN can be measured in whole blood for patients on oral doses of AZA or 6MP for IBD. This will allow blood to be frozen immediately following collection minimizing metabolite degradation and eliminating the need for

RBC separation and washing. Significant levels of 6TGN (1.1 nmol/8×10^8 cells, 31-fold more than in RBC) have been found in the neutrophils of renal transplant patients taking oral AZA, but not in any of the other cells present in blood other than erythrocytes [16]. Assuming there are 1000-fold more RBC than neutrophils in blood, the potential contribution from neutrophils to the concentration of 6TGN in blood would be approximately 3% of the total in this patient population. The levels of 6MMPN in neutrophils and lymphocytes are extremely low or undetectable [16] and therefore would not contribute significantly to the total concentration in blood. Unspecified concentrations of 6TG riboside and 6MMP have been detected, but not quantified, in plasma from a single patient with ALL during a high dose intravenous infusion of 6MP (1 g/m^2 over 6 h) [18]. 6MMP has been detected during a high dose intravenous infusion of 6MP (1.3 g/m^2, 24 h) in five children with non-Hodgkin lymphoma, but declined rapidly after termination of the infusion [17]. At 24 h after the end of the infusion, 6MMP was not present in any of the patients. 6MMP riboside reached a maximum concentration of 0.1–0.4 μM for three of these patients at the end of the 48 h study, but was never detectable in the other two. IBD patients typically receive oral doses of 6MP (1.0–1.5 mg/kg/day) that are 20 times less than those administered to cancer patients, apparently resulting in levels of 6TGN and 6MMPN in plasma that are too low to contribute significantly to the levels measured for RBC in blood.

In this work we also evaluated the stability of 6TGN and 6MMPN in a patient sample stored at approximately −80°C for 6 months (Table 2). The RBC 6TGN and 6MMPN concentrations in blood were measured on the same day the blood was collected and then after storage at approximately −80°C. The 6TGN concentration decreased 12% from 102±8 pmol/8×10^8 RBC at week 0 to 90±3 pmol/8×10^8 RBC after 24 weeks (Table 2). The levels of 6MMPN in the blood did not change significantly over the 24 weeks at approximately −80°C. Recovery of 6MMP in blood (59.4%±5.8, RSD=9.8, n=24) was determined by comparing direct injections of the 6MMP to three concentrations of 6MMP spiked blood that had been assayed. Recovery of 6MMP in RBC (38–41%) has been reported previously [12]. Sulfamethoxazole, the internal standard used for the 6MMP assay, was recovered at 82%±4, RSD=4, n=5.

Table 2
Stability of 6TGN and 6MMPN in blood[a]

Weeks at −80°C	6TGN (pmol/8×10^8 RBC)	6MMPN (pmol/8×10^8 RBC)
0	102±8	419±43
1	101±7	399±15
2	95±5	ND
3	ND	420±18
4	94±4	ND
8	94±1	ND
10	ND	415±33
15	ND	393±15
16	84±2	ND
24	90±3	428±14

[a] Blood from a patient receiving AZA (150 mg/day) for Crohn's disease was analyzed the day it was collected and then periodically for 24 weeks to monitor the stability of 6TGN and 6MMPN at approximately −80°C. Values are the mean±SD, n=5. ND=Not Determined.

It has been reported that 6MMPN are converted to a derivative during hydrolysis of nucleotides with perchloric acid at pH 0 [22]. Using the modified method of Erdmann et al. [12] in our investigation, 6MMPN were hydrolyzed with sulfuric acid at pH 0.8. Under these conditions, 6MMP was recovered and quantified by HPLC as described in the Experimental section. The hydrolysis product that was quantified as 6MMP in our investigation eluted at the same retention time (3.2 min) and had an identical UV spectrum (λ_{max}=290 nm) as authentic 6MMP (data not shown). Another peak in the hydrolysis mixture that eluted at 2.5 min and had a λ_{max}=305 nm may be the 6MMP derivative identified by Dervieux and Boulieu [22]. Apparently, the milder hydrolysis conditions used in our study resulted in only a partial conversion of 6MMP to this derivative.

Modifications of the assay for 6TGN, as described in the methods, eliminated the chromatographic interference at the retention time of 6TGN (Fig. 2). This improvement allowed more samples to be analyzed per day, more accurate determination of 6TGN in blood, and the quantification of 6TGN in human duodenal tissue. Recovery of 6TG in blood (55.5%±0.0, RSD=6.5, n=12) was determined by comparing direct injections of the 6TG sulfonate to

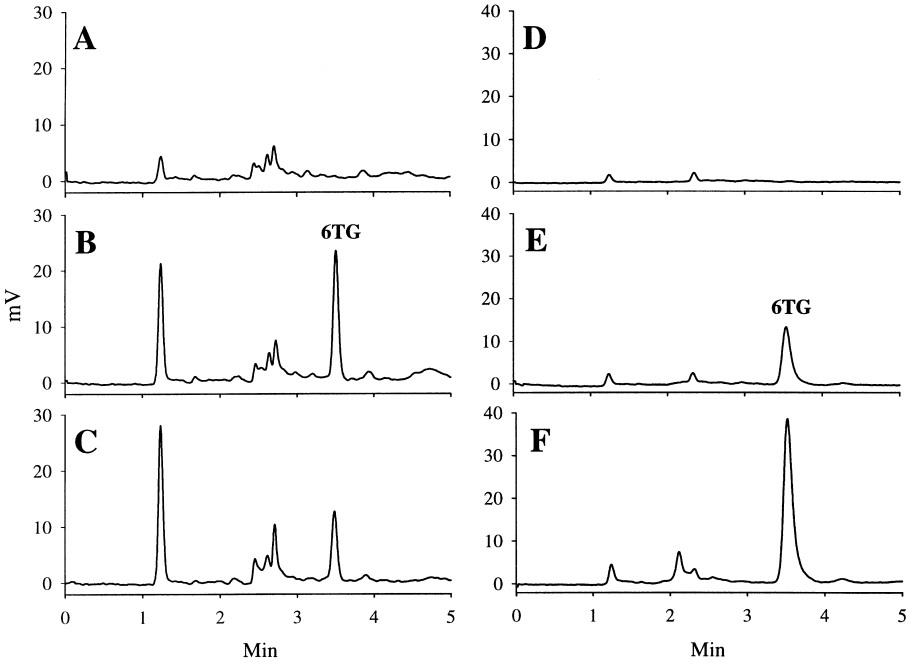

Fig. 2. HPLC–Fluorescence chromatograms show the improved assay and chromatography for measuring 6TGN in blood and intestinal (duodenal) biopsy tissue. (A) Duodenum from a patient not on 6MP or AZA therapy, (B) Duodenum (blank) spiked with 2.2 pmol 6TG/mg tissue, (C) Duodenum (1.2 pmol 6TGN/mg tissue) from a patient taking oral 6MP for Crohn's disease (75 mg/day), (D) Blood blank, (E) Blood spiked with 10 ng/100 μl 6TG, (F) Blood (288 pmol 6TGN/8×10^8 RBC or approximately 27 ng 6TGN/100 μl blood) from a patient on oral AZA maintenance therapy for Crohn's disease (100 mg/day). 1 mV=1 milli-flourescence unit (mFU).

three concentrations of 6TG spiked blood that had been assayed. Recovery of 6TG in RBC (74–81%) has been reported previously [12].

Standard curves for the improved assay demonstrated a linear relationship between peak areas and concentrations for 6TG in blood and RBC (Table 3). The concentrations of 6MMP in blood and RBC were also linear with peak areas of the analyte normalized to the peak area for the internal standard. The intra-batch ($n=5$) and inter-batch ($n=15$) precision and accuracy for the improved 6TG assay and 6MMP assay are summarized in Table 4. RSD for

Table 3
Typical values for 6TGN and 6MMP standard curves in multiple matrices[a]

	Slope	*y*-intercept	Correlation coefficient
6TGN	(mV/ng/100 μl)	(mV)	
Blood	30.9±0.2	−2.52±2.11	>0.999
RBC	27.9±0.4	−9.56±3.70	>0.999
Tissue	58.4±0.7	−0.55±1.06	0.999
6MMPN	$(ng/100\ \mu l)^{-1}$	(Peak Area Ratio 6MMP/IS)	
Blood	0.48±0.01	$1.17\times10^{-3}\pm1.44\times10^{-3}$	>0.999
RBC	0.47±0.01	$-1.76\times10^{-3}\pm2.17\times10^{-3}$	>0.999

[a] Values±SE for typical standard curves are shown as calculated by Sigma Plot Version 5.01 (SPSS Inc., Chicago, IL). The 6TG standard curves were constructed by plotting peak area for the 6TG standards versus their spiked concentration with 1/y weighting. The 6MMP standard curves were made by plotting the peak area ratio of 6MMP and internal standard versus the spiked 6MMP concentration, with 1/×weighting. For the 6TGN assay 1 mV=1 milli-fluorescence unit (mFU).

Table 4
Precision and accuracy of 6TG and 6MMP standards spiked into blood[a]

Standard	Spiked concentration (ng/100 μl)	Intra-batch (n=5)			Inter-batch (n=15)		
		Concentration (ng/100 μl) (mean±SD)	RSD (%)	Accuracy (%)	Concentration (ng/100 μl) (mean±SD)	RSD (%)	Accuracy (%)
6TG	5	5.1±0.1	1.2	101.7	4.9±0.3	5.4	97.4
	20	20.3±0.3	1.6	101.4	19.3±1.0	5.2	96.5
	40	40.3±1.0	2.6	100.7	38.8±1.5	3.9	97.0
6MMP	30	30.2±0.8	2.7	100.7	32.2±2.2	6.8	107.3
	200	199.7±3.9	2.0	99.8	201.7±8.4	4.2	100.8
	1000	894.7±11.8	1.3	89.5	933.8±69.5	7.4	93.4

[a] To determine the accuracy and precision for the assays, 5 ml frozen blood was spiked with 6TG or 6MMP and frozen at approximately −80°C in 1 ml aliquots. The spiked standards were analyzed on three separate days in replicates of five and compared to the standard curve for that day.

intra- and inter-batch analyses were <8% for both 6TG and 6MMP, and the accuracy ranged from 89.5 to 107.3% at known low, middle, and high concentrations.

The improved assay for 6TG in blood was used to determine the potential for measuring 6TG in human intestinal biopsy samples. Standard curves of 6TG spiked into blank intestinal tissue were linear, with a limit of quantification of 0.4 pmol 6TGN/mg intestinal tissue (Table 3). Using this method, we were able to quantify 6TG in the duodenum of an IBD patient receiving maintenance 6MP therapy (Fig. 2). Further validation of this assay in human intestinal tissue is necessary, but will require a much larger amount of intestinal biopsy tissue than was available for this study.

4. Conclusion

We have improved the current method of therapeutic drug monitoring for IBD patients receiving oral AZA or 6MP. The use of blood rather than washed RBC for 6TGN and 6MMPN determinations allows samples to be frozen immediately after they are collected, thus reducing analyte degradation and analysis time. Our stability study data show there is a 12% loss in 6TGN and no loss in 6MMPN in blood samples stored at approximately −80°C for 24 weeks. Finally, we have modified the assay for 6TGN to eliminate the blank chromatographic interference, allowing for quantification of 6TGN in human intestinal tissue. The improvements we have made to the methods for quantifying 6TGN in blood or tissue and 6MMPN in blood will reduce analysis time, increase the accuracy of metabolite measurements, and may be applicable to quantifying the active metabolites of AZA and 6MP in the target tissue of IBD patients.

Acknowledgements

We thank Dr. Jarmila Sulikova for her work on the 6TGN stability study at ambient temperature. This research was funded by Proctor & Gamble and by grant FDT-000-886 from the FDA.

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