Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC

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6-thioguanine (6-TGN) and methyl 6-mercaptopurine nucleotides (Me6-MPNs) are the two major metabolites found in erythrocytes after administration of azathioprine. In an attempt to understand the role of these metabolites in the pharmacologic and toxic activity of thiopurines, we have developed a HPLC method for the simultaneous determination of 6-TGNs and Me6-MPNs in erythrocytes. A simple and rapid treatment procedure based on deproteinization by perchloric acid with di-thiothreitol is described. The nucleotides were hydrolyzed to their own bases by heating the sample for 45 min at 100 °C. During acid hydrolysis Me6-MP was converted into a compound analyzed on a Purospher RP18-e column with 0.02 mol/L dihydrogenophosphate buffer–methanol as eluents. With this procedure, mean recoveries of 73.1% and 84.0% for 6-TGN and Me6-MPN derivatives, respectively, were found.

Azathioprine is an immunosuppressive drug coadministered with cyclosporine and corticoids to prevent rejection after transplantation [1]. Azathioprine, a prodrug of 6-mercaptopurine (6-MP), acts as an antimetabolite of purines after intracellular conversion by hypoxanthine guanine phosphoribosyl transferase into 6-thioinosine monophosphate (6-TIMP).3 This anabolic route leading to 6-thioguanine nucleotides (6-TGNs) is in competition in red blood cells (RBCs) with methylation of 6-TIMP into methyl 6-TIMP (Me6-TIMP) via thiopurine S-methyltransferase (TPMT), a cytosolic, S-adenosyl methionine-dependent enzyme. This enzyme exhibits a genetic polymorphism, which could explain a part of the wide interindividual variability of response to thiopurine compounds like azathioprine [2]. Administration of azathioprine in transplant patients leads rapidly to severe myelosuppression when the homozygote allele for TPMT deficiency is expressed [3]. Then, high 6-TGN concentrations in RBCs could be correlated with low erythrocyte TPMT activity, which exhibits a favorable clinical outcome in children with acute lymphoblastic leukemia treated by 6-MP [4]. However, the relation between high 6-TGN concentrations in RBCs and myelosuppression induced by azathioprine remains controversial [5].

More recently, Me6-TIMP was shown to strongly inhibit purine de novo synthesis in a concentration-dependent manner, leading to cytotoxicity in Molt F4 cells, suggesting that methyl 6-mercaptopurine nucleotides (Me6-MPNs) could explain part of the pharmacologic activity of azathioprine [6].

Few HPLC methods have been developed for the determination of thiopurine and methylated thiopurine metabolites of azathioprine or 6-MP in biological fluids [7–9]. Lennard and Singleton [7] developed a reversed-phase liquid chromatographic method on Resolve C18 for the simultaneous determination of 6-TG, 6-MP, and Me6-MPNs in erythrocytes. The free bases, 6-TG and 6-MP, liberated from the respective nucleoside and nucleotide moiety by acid hydrolysis, were analyzed on an octadecylsilane column.

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3 Nonstandard abbreviations: (Me)6-MP(N), (methyl) 6-mercaptopurine (nucleotide); (Me)6-TIMP, (methyl) 6-thioinosine monophosphate; 6-TG(N), 6-thioguanine (nucleotide); RBC, red blood cell; TPMT, thiopurine S-methyltransferase; PMA, phenylmercuric acetate; and DTT, dithiothreitol.

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column after liquid–liquid extraction with dichloromethane. Me6-MP and Me6-TG were extracted from RBCs with ethyl acetate and then analyzed on a cyanopropylsilane column. The recoveries were <40% for Me6-MPNs and 60% for Me6-TGNs.

More recently, an anion exchange method for the determination of intracellular mono-, di-, and triphosphate thiopurine and methylthiopurine nucleotides in RBCs was described by Keuzenkamp-Jansen et al. [9]. Measurement of concentrations of TGNs in RBCs was performed after extraction with methanol and methylene chloride, followed by oxidation of TGNs with permanganate. Methyl thioinosine nucleotides were extracted with perchloric acid and dipotassium hydrogen phosphate. Previously, we reported a method for the determination of 6-TGNs, 6-MPNs, and 6-thioxanthine nucleotides in erythrocytes on a Hypersil ODS 3-μm column with a gradient elution mode with 0.02 mol/L KH2PO4 (pH 3.5):methanol (40:60 by vol) as eluents [10]. The sample treatment procedure was simple, involving perchloric acid deproteinization with dithiothreitol (DTT) and hydrolysis of thiopurine nucleotides into their free bases by heating of the acid extract [10]. In an attempt to understand the role of the methylated thiopurine nucleotides vs thiopurine nucleotides in the pharmacologic and (or) toxic effect of thiopurine drugs like azathioprine, we report a reversed-phase HPLC method with a simple and rapid sample treatment procedure for the analysis of 6-TGNs and Me6-MPNs in RBCs in a single run.

Materials and Methods

Reagents and Stock Solutions
6-TG, Me6-MP, and DTT were obtained from Sigma. Methanol, potassium dihydrogenophosphate, and perchloric acid were obtained from Merck.

For experiments, stock solutions were prepared in 0.1 mol/L HCl and stored at −80 °C. All solutions were stable at −80 °C for 4 weeks.

Apparatus and Chromatographic Conditions
The liquid chromatograph consisted of a model 510 pump connected with photodiode array detector model 960 and Wisp 715 solvent delivery model (all from Waters).

6-TG and Me6-MP derivative liberated from the nucleotide moiety were analyzed by a reversed-phase HPLC method. The separation was performed on a Purospher RP 18-e column, 5-μm particle size, protected by a Purospher RP 18-e guard column (from Merck) with a linear gradient elution mode with 0.02 mol/L potassium phosphate (pH 3.5) and 0.02 mol/L potassium phosphate (pH 3.5):methanol (40:60 by vol). The concentration of methanol varied from 0 to 200 mL/L over a period of 12 min. The flow rate was 1.2 mL/min. Detection of 6-TG and Me6-MP derivative was performed at 341 nm and 304 nm respectively. Peak identity was confirmed through library matching by comparison of unknown peak to reference spectra of calibrator. All analyses were performed at ambient temperature. The purospher RP 18-e column has demonstrated a long lifetime: ~600 samples were injected into the column without any deterioration of its performance.

Sample Collection and Storage
Blood samples (5 mL) were collected into heparinized tubes containing 1 mg of DTT and centrifuged without delay at low temperature (4 °C). Hematocrit and erythrocyte counts were obtained from each sample. Plasma was decanted, and the leukocytes and the upper layer of erythrocytes were removed. Drug concentrations were normalized to 10⁸ RBCs. The remaining erythrocytes were stored at −80 °C until analysis.

Sample Treatment
Of the remaining erythrocytes, 500 μL were transferred into a tube containing 5 mg of DTT and the totality was rapidly deproteinized by 50 μL of 700 mL/L perchloric acid. The deproteinized samples were centrifuged at 3000g for 15 min at 4 °C. The supernatants were removed and the acid extracts were then heated for 45 min at 100 °C to hydrolyze thiopurine nucleotides into their bases. After cooling, a 80-μL aliquot was injected into the column. All assays were run in duplicate.

Results and Discussion

Chromatographic Separation
Retention behavior of 6-TG and Me6-MP derivative was not significantly modified by the pH of the mobile phase in the range 3.0 to 7.0. Likewise, the ionic strength did not significantly change the retention of the compounds. In contrast, the retention behavior can be strongly influenced by the concentration of organic modifier (methanol). A gradient elution mode from 0 to 200 mL/L methanol in 12 min was optimal to elute 6-TG and to separate the Me6-MP derivative from DTT.

The chromatogram of the erythrocyte sample supplemented with 6-TG and Me6-MP derivative is presented in Fig. 1.

Recoveries
Analytical recoveries were determined by adding known concentrations of compounds to erythrocytes and comparing peak heights with those obtained by direct injection of aqueous calibrators. For the determination of recoveries of the Me6-MP derivative, 500 μL of aqueous calibrator of Me6-MP was heated for 45 min at 100 °C with 50 μL of 700 mL/L perchloric acid to allow the conversion of Me6-MP into its derivative. The conversion is complete under these conditions.

Deproteinization Step
Previously we reported that the recoveries of 6-thiopurines like 6-TG were greatly influenced by the addition of DTT during the sample treatment procedure [10]. DTT protects thiol function from oxidation during the depro-
teinization step but did not contribute to an improvement of recoveries for Me6-MP, the thiol group being protected from oxidation by a methyl function.

**HYDROLYSIS STEP**
The recovery of Me6-MP is greatly influenced by the pH of acid hydrolysis. As shown in Fig. 2, Me6-MP was converted into a derivative compound when the pH was <1.0. The determination of Me6-MP per se appeared technically difficult because of the instability of this compound at a pH value required for the hydrolysis of thiopurine nucleotides into their free bases.

The conversion of Me6-MP into the derivative during the heating step under the acidic conditions described above was 100%. Mean analytical recoveries of Me6-MP derivative and 6-TGN were 84.0% and 73.1% respectively. No significant decrease in 6-TGN and Me6-MPN derivative concentration was found after a storage of 24 h at room temperature.

**LINEARITY AND PRECISION**
The relation between peak height and concentration was defined by using eight concentrations: 0.3, 0.6, 1.5, 3.0, 6.0, 10.0, 30.0, and 50.0 μmol/L packed cells for 6-TGN and 1.5, 3.0, 6.0, 15.0, 30.0, 60.0, 90.0, and 120 μmol/L packed cells for Me6-MP derivative. The calibration curves were linear, with a correlation coefficient >0.998 for each compound. The typical regression equations were $y = 1960x - 233$ for 6-TGN and $y = 2946x - 641$ for Me6-MPN derivative. The CVs for the calibration curve ranged from 5.3% to 9.0% for 6-TGN and from 4.0% to 9.8% for Me6-MPN derivative. The minimal amount detectable, at a signal-to-noise ratio of 4, was 1.8 pmol for 6-TGNs and 6.7 pmol for Me6-MPNs. The quantification limit was 0.1 μmol/L packed cells for 6-TGN and 0.3 μmol/L packed cells for Me6-MP derivative, with a CV <15% for a 500-μL sample volume. The intraassay and interassay precision and accuracy, determined by replicate analyses of erythrocyte samples supplemented with the compounds of interest, are given in Table 1.

**SELECTIVITY**
No interference with the related endogenous compounds uric acid, hypoxanthine, xanthine, guanine, guanosine, or those commonly coadministered drugs in transplant recipients such as cyclosporine, azathioprine, ganciclovir, acyclovir, prednisone, or dexamethasone was found. Furthermore, 6-MP, 6-thioxanthine, and Me6-TG were separated from the compounds of interest. Chromatographic behavior of the compounds was investigated before and after perchloric acid hydrolysis.

**ANALYSIS OF PATIENT SAMPLES**
The method developed was used for the determination of thiopurine and methylated thiopurine nucleotide content...
in erythrocytes from lung or heart/lung transplant patients under azathioprine therapy.

Chromatograms of a blank erythrocyte sample and an erythrocyte sample from a patient who received azathioprine (2.0 mg/kg per os daily) is presented in Fig. 3. The concentration of thiopurine nucleotides found was 48 pmol/10⁸ cells (4.6 μmol/L packed cells) for 6-TGNs and 1042 pmol/10⁸ cells (99.9 μmol/L packed cells) for Me6-MPNs.

A wide range, from nondetectable to 290 pmol/10⁸ cells, of 6-TGN concentrations was found in transplant patients receiving a mean daily dose of 2.0 mg/kg of azathioprine [5, 7, 8, 11]. Few data on Me6-MPN concentrations after azathioprine therapy are available [7]. 6-MPN concentrations found ranged from 1.7 pmol/8 × 10⁸ cells to 20.9 pmol/8 × 10⁸ cells (212 to 2612 pmol/10⁸ cells) [7].

The method described is selective and sensitive enough to analyze 6-TGNs and Me6-MPNs of azathioprine in erythrocytes in a single run. The sample treatment procedure described is rapid and simple and exhibits recoveries >84% for Me6-MP compared with previous reversed-phase published data. Moreover, the use of a Purospher column contributed to improve the peak symmetry of the Me6-MP derivative.

The identification of the Me6-MP derivative by HPLC–mass spectrometry is under way in our laboratory.

### Table 1. Precision and accuracy of thiopurine compounds in erythrocytes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration added, μmol/L packed cells</th>
<th>Concentration found, μmol/L packed cells</th>
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<td>Intraassay (n = 5)</td>
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<tr>
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![Fig. 3. Chromatograms of a blank erythrocyte sample (A) and an erythrocyte sample (B) from a patient receiving 2.0 mg/kg daily of azathioprine. Peaks: 6-TGN, 48 pmol/10⁸ cells; Me6-MPN, 1042 pmol/10⁸ cells.](image-url)
We thank Martine Sauviat for his excellent technical assistance.

References


