An improved HPLC method for the quantitation of 6-mercaptopurine and its metabolites in red blood cells

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Abstract

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A procedure is described for the rapid determination of the intra-erythrocyte concentration of 6-mercaptopurine (6-MP) and its metabolites, 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP). Erythrocytes (8×10^8 cells) in 350 µl Hanks solution containing 7.5 mg dithiothreitol were treated with 50 µl 70% perchloric acid. The precipitate was removed by centrifugation (13,000 g) and the supernatant hydrolyzed at 100°C for 45 min. After cooling, 100 µl was analyzed directly by HPLC using a Radialpack Resolve C18 column eluted with methanol-water (7.5:92.5, v/v) containing 100 mM triethylamine. 6-TG, 6-MP and the hydrolysis product of 6-MMP, 4-amino-5-(methylthio)carbonyl imidazole, were monitored at 342, 322 and 303 nm using a Shimadzu SPD-M10A diode array UV detector. The analytes eluted at 5.3, 6.0 and 10.2 min, respectively. The calibration curves were linear ($r^2 > 0.998$), and the analytical recoveries were 73.2% for 6-TG, 119.1% for 6-MP and 97.4% for 6-MMP. The intra- and inter-assay variations were highest for 6-MP (9.6 and 14.3%, respectively). The lowest detectable concentrations were 3, 3 and 25 pmol/8×10^8 erythrocytes for 6-TG, 6-MP and 6-MMP, respectively. The quantification limits (coefficients of variation <15%) were 8, 10 and 70 pmol/8×10^8 erythrocytes for 6-TG, 6-MP and 6-MMP, respectively. The method was applied to the analysis of 183 samples from 36 children under chemotherapy for acute lymphoblastic leukemia. The concentrations of the metabolites in the red cells of the patients ranged from 0 to 1934 pmol/8×10^8 erythrocytes for 6-TGN, and from 0 to 105.8 and 0 to 45.9 nmol/8×10^8 erythrocytes for 6-MP and 6-MMP, respectively. The procedure gave results that were in agreement with those obtained with other methods designed to detect cases of non-compliance with treatment, including patient interviews and medical evaluation, among others, demonstrating its applicability to monitoring the treatment of leukemic children.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy affecting children. Prior to the discovery and application of chemotherapeutic agents, the majority of children suffering from ALL survived for only one or two months after diagnosis (1). However, over the last 30 years there has been a significant improvement in the prognosis of such patients and, currently, 70 to 80% of recently diagnosed children in developed countries present disease-free survival for more than 5 years, and most will eventually be cured (2). Treatment failures may be attributed to several factors, a major one being lack of adhesion to the prescribed treatment (3). Procedures to assess compliance with treatment, especially with respect to the effective delivery of chemotherapy, are of great importance to ALL patients and can even determine the outcome of the disease. Such assessments may be carried out by various indirect methods including patient interviews, medical evaluation, drug audits, etc. (4), although more direct methods involving quantitation of the drug and its metabolites in urine and erythrocytes have been proposed (5-9).

6-Mercaptopurine (6-MP), a chemotherapeutic agent commonly used to treat ALL, is an analogue of the natural nucleoside hypoxanthine. As is the case for the majority of purine analogues, 6-MP is a pro-drug and must be converted into the nucleotide by intracellular enzymes in order to exert its cytotoxic effects (10). Such conversion after oral administration of the drug follows three competing routes (Figure 1) (11,12). One route occurs both in the intestinal mucosa and in the liver and involves the enzyme xanthine oxidase, which transforms 6-MP into thiouric acid, an inactive metabolite (10). A second route generates the active metabolite 6-thioinosine monophosphate (TIMP) by the action of the enzyme hypoxanthine-guanine phosphoribosyltransferase (10). This transformation results in the formation of active nucleotides including nucleotides of 6-thioguanine (6-TGN). The incorporation of 6-TGN into DNA and RNA is considered to be the principal mechanism of action of 6-MP (13). The third route is catalyzed by the enzyme thiopurine methyltransferase (TPMT), which converts 6-MP into 6-methylmercaptopurine (6-MMP) (13). Despite some views to the contrary, this methylation is considered to be an inactivation mechanism since 6-MMP seems to be inactive (13). However, while the metabolism of 6-MP by TPMT reduces the formation of active 6-TGN (14), this enzyme is also responsible for the formation of methyl-TIMP, a metabolite that seems to be important for the cytotoxic activity of 6-MP as it may inhibit the *de novo* synthesis of purines (13). Significant differences in the activity of TPMT may be observed between individuals, thus accounting for the reported (15) differences in susceptibility to treatment with 6-MP. Studies among different populations and families have demonstrated a genetic autosomal co-dominant polymorphism of TPMT: thus, ca. 90% of Caucasians and Afro-Americans present high enzymatic activity, 10% present intermediate activity, and 1 in 300 individuals inherits deficient TPMT activity (16,17).

The most important metabolites of 6-MP, the 6-TGNs, slowly accumulate in red cells and reach a steady-state concentration within
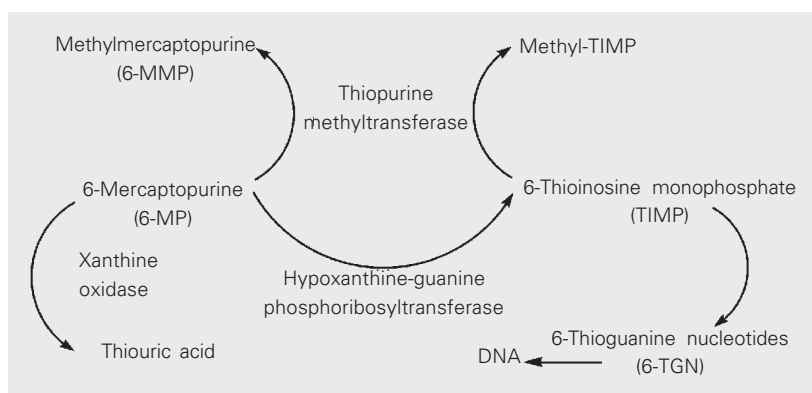

Figure 1. Schematic representation of the metabolism of 6-mercaptopurine (adapted from Refs. 10 and 37).

1 to 4 weeks after the beginning of treatment (18). These metabolites are rapidly incorporated into the DNA and RNA of leukemic cells, leukocytes and their precursors in the bone marrow, thus making their detection somewhat difficult (19). However, the erythrocytes act as reservoirs of these metabolites since they do not synthesize nucleic acids, and the intra-erythrocyte TGN concentration, which reflects this accumulation following multiple doses of 6-MP (20), has been proposed as a useful parameter with which to monitor 6-MP maintenance chemotherapy (21-23). It should be emphasized, however, that this parameter alone may not be sufficient to optimize the treatment of some patients with ALL, and the concomitant evaluation of TPMT activity and leukocyte levels has been recommended in order fully to monitor the chemotherapy (24).

The majority of the methods for the quantitation of 6-MP metabolites involve the hydrolysis of nucleotides to the corresponding bases that can then be separated by HPLC and detected by UV absorption. During hydrolysis, 6-MMP is converted completely into 4-amino-5-(methylthio)carbonyl imidazole (AMTCI) (25) which, unlike 6-MMP, can be quantified by HPLC-UV under the same conditions as employed for the bases: the concentration of AMTCI thus determined correlates directly with that of the 6-MMP originally present (26).

We describe a quantitation procedure based on the publications of Dervieux and Boulieu (27) and Lennard and Singleton (26), which permits the rapid quantitation of the intra-erythrocyte levels of 6-MP, 6-TGN and 6-MMP. The method has been fully validated and employed to monitor 36 children with ALL receiving chemotherapy with 6-MP.

Material and Methods

Chemicals

Dithiothreitol (DTT), 6-thioguanine (6-TG), 6-MP, and 6-MMP were purchased from Sigma; perchloric acid was from Carlo Erba and Hank's balanced salt solution was from Gibco BRL; triethylamine, phosphoric acid and all other chemicals were purchased in analytical grade from Merck (Rio de Janeiro, RJ, Brazil). Grade I water was produced from glass-distilled water using a Milli-Q-UF Plus (Millipore, Molshein, France) system. All glassware was treated with 30% nitric acid for at least 12 h and then rinsed thoroughly with water.

Patient selection

The project was approved by the Ethics Committee of Research on Human Beings of the Federal University of Minas Gerais (COEP-UFMG 007/97). The children were included in the study only after their parents or guardians had been fully informed and had signed the terms of consent. The study population consisted of 36 patients aged less than 18 years, who had been diagnosed at UFMG from May 1997 to December 2000 to be suffering from ALL (as assessed by a bone marrow smear stained with May-Grünwald-Giemsa and confirmed by immunophenotyping) and had started the maintenance phase of the Brazilian therapeutic protocol GBTLI-93. The recommended dosage of 6-MP for this group of patients was 50 mg m^{-2} day^{-1} but was adjusted for each child according to the leukocyte count and the presence of clinically relevant infections: retrospectively, the mean dosage prescribed ranged from 24.6 to 66.9 (median 46.1) mg m^{-2} day^{-1}. Blood samples for the assay of 6-MP metabolites were collected from the eighth week of the maintenance phase of treatment and at intervals of approximately 8 weeks thereafter. Patients should have been under continuous administration of 6-MP for a minimum period of 4 weeks and should not have received packed red cell transfusions in the 6 weeks preceding blood sample collection. Two to six samples were ob-

tained from each patient (providing a total of 183 samples for the entire population) according to the extent of the maintenance phase of treatment. The samples were coded and the identities of the patients were not revealed until the quantitation data and the answers given in the patients' questionnaires were analyzed at the same time.

Preparation of clinical samples

Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) as anticoagulant, and red blood cells were separated by centrifugation at 160 g for 10 min at 4ºC. The platelet-rich supernatant plasma and the buffy coat containing leukocytes and platelets were discarded, and the pellet was washed with two volumes of Hanks solution and centrifuged at 160 g for 10 min at 4ºC. Again, the supernatant was discarded and the erythrocytes were suspended in Hanks solution at a density of 8 x 10^8 erythrocytes/ 200 µl. This suspension was kept at -20ºC until required for further processing (28).

Preparation of standard mixtures

Stock solutions of 6-TG and 6-MMP at concentrations of 185.6 and 242.4 µg/ml, respectively, were prepared by dissolving the appropriate amount of weighed standard in 1 ml of 0.1 M NaOH, diluting with 21 ml water and acidifying with 2.5 ml of 0.1 M HCl. A stock solution of 208.0 µg/ml 6-MP was prepared by dissolving the appropriate amount of weighed standard in 1 ml of 0.1 M NaOH and completing the volume to 25.0 ml with water. The stock solutions were used to prepare six standard mixtures with different concentrations of metabolites by dilution as appropriate with 0.1 M HCl containing 154 mg/ml DTT. Aliquots (1 ml) of these standard mixtures were transferred to 1.5 ml microcentrifuge tubes and stored at -20ºC.

Preparation of standard samples

A 50-µl aliquot of each of the six standard mixtures (see Table 1) was added to 200 µl Hanks solution containing 8 x 10^8 erythrocytes obtained from healthy volunteers (who had been taking no medications) and processed as described for the clinical samples. The final concentrations of the metabolites in each of the six standard samples are shown in Table 1.

Sample extraction and hydrolysis

A procedure based on a published protocol (27) was employed. The erythrocyte suspension (200 µl), together with DTT (100 µl of solution containing 75 mg/ml) and water (50 µl) were added to an Eppendorf tube

Table 1. Precision and accuracy of the determination of intra-erythrocyte concentrations of 6-MP, 6-TG and 6-MMP.

Metabolites in standard samples	Amount added (pmol)	Amount found (pmol) (% of amount added)	Coefficient of variation (%)	
			Intra-assay (N = 3)	Inter-assay (N = 10)
6-MP				
1	200	207 (104)	5.6	–
2	160	164 (103)	1.2	10.4
3	120	118 (98)	9.6	–
4	80	78 (98)	1.9	14.3
5	40	40 (100)	7.8	13.6
6	20	23 (115)	1.1	–
6-TG				
1	1230	1222 (99)	5.9	–
2	820	830 (101)	2.9	4.0
3	410	424 (103)	2.6	–
4	246	239 (97)	5.4	2.1
5	164	166 (101)	4.1	7.5
6	82	89 (108)	0.9	–
6-MMP				
1	24000	24340 (101)	5.2	–
2	12000	12150 (101)	1.4	6.2
3	6000	6070 (101)	4.6	–
4	3000	2970 (99)	1.0	6.7
5	1500	1520 (101)	5.5	14.3
6	750	830 (110)	4.0	–

The erythrocytes (8 x 10^8), obtained from healthy volunteers, were suspended in 200 µl Hanks solution and spiked with the indicated amount of the metabolites. Extraction, hydrolysis and HPLC analysis were performed as described in Methods. The amount found was back-calculated from the calibration curve. For abbreviations, see Figure 1.

held in an ice bath. Following homogenization, the suspension was treated with 70% perchloric acid (50 µl), mixed with a vortex mixer for 30 s, and centrifuged at 13,000 *g* for 15 min at room temperature. The supernatant (300 µl) was transferred to a 2-ml vial closed with a Teflon-lined screw cap, and heated at 100ºC for 45 min to hydrolyze the nucleotides and also to generate AMTCI from 6-MMP (25). Hydrolyzed samples were stored in an ice bath, and aliquots (100 µl) were injected into the HPLC apparatus for analysis as described below.

Chromatographic assay

Standard mixtures, standard samples and clinical samples were analyzed by HPLC (26) using a Shimadzu HPLC system consisting of an LC-10AD solvent delivery pump, an FCV-10AL low pressure solvent selector valve and an SPD-M10A diode array UV detector: a Waters Radial-Pak Resolve C18 column (100 x 8 mm; 5-µm particle size) was mounted in a Waters RCM 8 x 10 radial compressor and protected by a Waters Guard-Pak Resolve C18 pre-column. Elution was with methanol-water (7.5:92.5, v/v) containing 100 mM triethylamine and adjusted to pH 3.2 with 0.1 M phosphoric acid: immediately before analysis, DTT was added to the eluent to produce a 0.5-mM solution that was purged with helium and pumped at a flow rate of 1.4 ml/min. The column effluent was monitored simultaneously at 303 nm for AMTCI, at 322 nm for 6-MP and at 342 nm for 6-TG. The calibration curves and quantifications were based on the peak areas as determined using provided Shimadzu software (Class LC-10). After each day of use, the analytical column was washed with 60 ml of methanol and the guard-column was replaced after 300 injections.

Assay validation

Standard samples were used to deter-

mine the analytical recovery, accuracy and precision of the method over the entire concentration range. The intra-assay variation was calculated from triplicate measurements at each concentration, while the inter-assay variation was obtained from experiments completed on 10 different days. The analytical recovery was calculated by comparing the HPLC peak areas of processed standard samples with processed and unprocessed standard mixtures.

Results

HPLC chromatography

Figure 2A shows an HPLC chromatogram of a processed blood sample obtained from an untreated healthy volunteer: after the first 4.3 min of elution, no peaks could be detected at 303, 322 or 342 nm. Figure 2B depicts the HPLC chromatogram of a processed standard mixture showing peaks of 6-TG (detected at 342 nm), 6-MP (at 322 nm) and AMTCI (at 303 nm; corresponding to 6-MMP in the original mixture) eluting at 5.3, 6.0 and 10.2 min, respectively. The limits of detection (signal-to-noise ratio of 5) were 3 pmol for 6-TG and 6-MP, and 25 pmol for AMTCI/6-MMP, whereas the limits of quantification (LOQ; coefficient of variation <15%) were 8 pmol for 6-TG, 10 pmol for 6-MP, and 70 pmol for AMTCI/6-MMP). Values below the LOQ were interpreted as showing the absence of the metabolite. In order to determine the shelf life of the standard mixtures, the mixtures were stored at -20ºC during the study period (20 months) and showed unchanged HPLC profiles when re-analyzed after this time.

Assay validation

The calibration curves constructed using standard samples were linear and the correlation coefficients (r^2) were 0.9992, 0.9904 and 0.9998 for 6-TG, 6-MP and AMTCI/6-

MMP, respectively: typical equations were, respectively, $y = 146.4x$, $y = 210.2x + 3378.3$ and $y = 115552x + 215099$, where y is the area under the peak and x the concentration of metabolite (pmol/8 x 10^8 erythrocytes). The intra-assay coefficients of variation
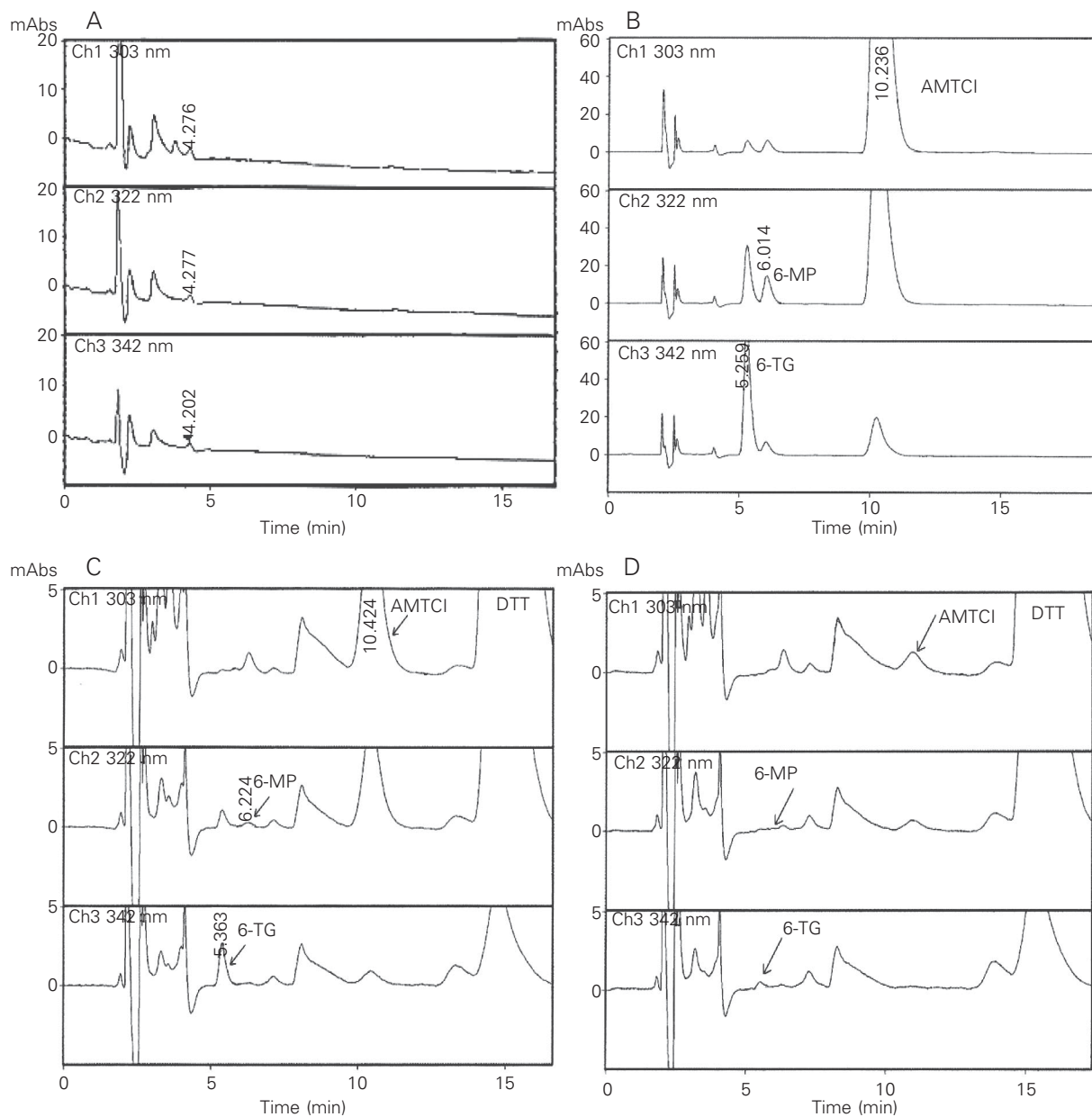

Figure 2. *A*, HPLC elution diagrams of a blood sample obtained from a healthy volunteer which was processed and then analyzed by HPLC using a Radial-Pak Resolve C18 column (100 x 8 mm; 5 µm particle size, eluted with methanol-water (7.5:92.5, v/v) containing 100 mM triethylamine adjusted to pH 3.2 with phosphoric acid; the flow rate was 1.4 ml/min; detection was by a photodiode array with channel 1 at 303 nm, channel 2 at 322 nm, and channel 3 at 342 nm. *B*, HPLC chromatogram of standard mixture 2 (see Table 1). Sample processing and HPLC analysis were as described in *A. C*, HPLC chromatogram of a blood sample collected from a patient using 6-MP at a dose rate of 47.8 mg m^{-2} day^{-1}. Sample processing and HPLC analysis were as described in *A. D*, HPLC chromatogram of a blood sample from the same patient as in *C* who had missed taking 6-MP at a dose rate of 58.1 mg m^{-2} day^{-1}, showing low levels of the metabolites 6-TG and MMP (AMTCI). Sample processing and HPLC analysis were as described in *A*. AMTCI = 4-amino-5-(methylthio)carbonyl imidazole; DTT = dithiothreitol. For other abbreviations, see Figure 1.

ranged from 0.9 to 9.6% and inter-assay coefficients of variation were 2.1 to 14.3%. When compared with the processed standard mixture, the recoveries of the metabolites in processed standard samples were 73.2% for 6-TG, 119.1% for 6-MP and 97.4% for AMTCI/6-MMP: such recoveries were obtained only after the amount of DTT recommended to be added to the sample in the published protocol (27) was doubled. Prior to the analysis of each batch of clinical samples, the standard curve was validated using the standard samples 2, 4 and 5 (see Table 1) employed as quality controls for the method.

Quantitation of metabolites in clinical samples

The intra-erythrocyte concentrations of 6-TGN determined in the blood samples of patients varied from 0 to 1934.0 (median 319.7; mean 372.1) pmol/8 x 10^8 erythrocytes, from 0 to 105.8 (median 2.9; mean 13.9) pmol/8 x 10^8 erythrocytes for 6-MP, and from 0 to 45.9 (median 6.3; mean 8.3) nmol/8 x 10^8 erythrocytes for 6-MMP. Figure 2C illustrates the HPLC chromatogram of a sample obtained from a patient using 6-MP at a dose of 47.8 mg m^{-2} day^{-1}. The peaks at 5.4 and 10.4 min correspond to 331.8 pmol 6-TGN/8 x 10^8 erythrocytes and to 4.7 nmol 6-MMP/8 x 10^8 erythrocytes, respectively. The intense peak at 14.8 min is due to the DTT added to the sample. Figure 2D shows the HPLC chromatogram of the same patient having failed to take a prescribed dose of 58.1 mg 6-MP m^{-2} day^{-1}; the peak at 5.4 min corresponds to 73.4 pmol 6-TGN/8 x 10^8 erythrocytes while the peak at 10.4 min corresponds to 0.3 nmol 6-MMP/8 x 10^8 erythrocytes. The peaks at 6.2 min, corresponding to 6-MP, could not be quantified in either sample as they were below the LOQ. The non-compliance of this patient with the treatment indicated by the above result was confirmed by interviews and reports from medical charts (29).

Discussion

Several methods have been reported for the determination of 6-MP and its metabolites in biological fluids (30). The ideal method should allow the assessment of patient compliance with the treatment, together with measurement of the absorption, transport and accumulation of the cytotoxic metabolites (31). Studies concerning the quantification of 6-MP and its metabolites in the urine of patients showed that the main disadvantage of this type of sample is that it can detect failures in adherence to the treatment only when the analysis is performed shortly after the missed event (6). On the other hand, the quantitation of 6-MP and its metabolites in erythrocytes is more appropriate for the assessment of the regular use of the drug during long-term treatment as a steady state is reached (32) and should then be maintained.

In a method proposed by Lennard and Singleton (26), the nucleotides in a sample of erythrocytes are hydrolyzed and the bases extracted with phenyl mercuric acetate (PMA) and toluene. The hydrolysis method transforms the non-HPLC quantifiable metabolite 6-MMP to the quantifiable derivative AMTCI (25). However, the PMA-based procedure has several disadvantages in that it is laborious, uses a highly toxic reagent, and is not robust enough for use in many laboratories (27,33). It was concluded (34) that the complexity of the technique poses serious difficulties to its use in large scale investigations. An alternative procedure (27) for hydrolysis and extraction was based on the use of 70% perchloric acid as both a deproteinizing and hydrolyzing agent, followed by direct injection into an HPLC apparatus and subsequent analysis. However, the chromatographic conditions used in the PMA-based procedure (26) are superior to those employed in the latter methodology in

that they permit the simultaneous determination of 6-MP, 6-TG and AMTCI/6-MMP in erythrocytes. In the present study, we combined the extraction and hydrolytic procedure developed by Dervieux and Boulieu (27) with the HPLC protocol published earlier (26). The modified procedure permitted the simultaneous analysis of 6-MP and its metabolites 6-TGN and 6-MMP in small-volume blood samples collected from children with ALL undergoing chemotherapy. The samples could be processed and analyzed by HPLC with isocratic elution in just 15 min, thus making the procedure appropriate for the investigation of a large number of samples. Another possibility, not examined in the present study, is the suitability of the method for automation with the aim of increasing the reproducibility and the throughput of the assay.

The calibration curves for 6-MP, 6-TG and AMTCI/6-MMP were linear, with correlation coefficients greater than 0.998. The intra-assay coefficients of variation were <10% over the entire concentration range for all of the compounds analyzed, while the inter-assay variations were <15% for 6-MP and AMTCI/6-MMP and below 7.5% for 6-TG. Previous reports (26,27) cite intra- and inter-assay variations below 10% for both 6-TG and 6-MMP at the two concentrations evaluated; the value for 6-MP, however, was not provided (26). In an earlier study (35), an intra-assay variation of more than 10% was reported for at least one point on the calibration curve for both 6-MP and 6-MMP. In the case of 6-TG, the variation was less than 10% at all points on the curve while the inter-assay variation was greater than 10% at one of the three concentrations monitored.

The analytical recoveries determined in the present investigation were 73.2% for 6-TG and 97.4% for 6-MMP, these values being similar to the previously reported values of 73.1 and 84% (27), 68.6 and 45% (26) and 74 and ≥38% (35), respectively. The LOQ values of 8, 10 and 70 pmol in 8×10^8 erythrocytes/200 µl respectively determined for 6-TG, 6-MP and 6-MMP in this study, are slightly better than those obtained earlier (26) and those reported in Ref. 35 (30 pmol for 6-TG and 120 pmol for 6-MMP). Other workers (34) have been able to achieve LOQ values of 18 pmol for both 6-TG and 6-MMP.

Our results show large differences in the intra-erythrocyte concentrations of 6-TG and 6-MMP in the samples analyzed. However, these intra- and inter-individual variations are similar to those reported by others (8,9,26,36). The inter-individual differences seem to reflect individual differences in 6-MP absorption and metabolism (due in part to the genetic polymorphism of TPMT) resulting in different bioavailability and distribution of the active metabolites in patients receiving similar doses. The variations observed in the values obtained for the same patient during a period of treatment suggest alternative explanations, among them the inadequate use of the prescribed medication (7) and non-compliance with treatment. Indeed, the aspect of non-compliance was also detected through questionnaires, interviews, and medical records (29), corroborating the observed low levels of drug and metabolites in erythrocytes of specific patients, and reinforcing the usefulness of this method in monitoring the treatment of children with ALL.

Acknowledgments

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