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Development and validation of an HPLC method for the rapid and simultaneous determination of 6-mercaptopurine and four of its metabolites in plasma and red blood cells

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ABSTRACT

An HPLC method has been developed and validated for the rapid determination of mercaptopurine and four of its metabolites; thioguanine, thiouric acid, thioxanthine and methylmercaptopurine in plasma and red blood cells. The method involves a simple treatment procedure based on deproteinisation by perchloric acid followed by acid hydrolysis and heating for 45 min at 100 °C. The developed method was linear over the concentration range studied with a correlation coefficient >0.994 for all compounds in both plasma and erythrocytes. The lower limits of quantification were 13, 14, 3, 2, 95 pmol/8×10^8 RBCs and 2, 5, 2, 3, 20 ng/ml plasma for thioguanine, thiouric acid, mercaptopurine, thioxanthine and methylmercaptopurine, respectively. The method described is selective and sensitive enough to analyse the different metabolites in a single run under isocratic conditions. Furthermore, it has been shown to be applicable for monitoring these metabolites in paediatric patients due to the low volume requirement (200 μl of plasma or erythrocytes) and has been successfully applied for investigating population pharmacokinetics, pharmacogenetics and non-adherence to therapy in these patients.

1. Introduction

Thiopurine drugs, which include 6-mercaptopurine (6-MP) and azathioprine (AZA), were introduced into clinical practice more than 50 years ago. They are used in the treatment of several diseases including childhood acute lymphobalstic leukaeima (ALL) [1] and inflammatory bowel disease (IBD) [2]. AZA (1, Fig. 1) is a nitroimidazole derivative of 6-MP (2, Fig. 1) [3]. Both, however, are inactive prodrugs, requiring transformation by hypoxanthine phosphoribosyl transferase (HPRT) into thioinosine monophosphate (TIMP) and subsequently into thioguanine nucleotides (6-TGNs) which mediate their pharmacological activity via incorporation into DNA and RNA (Fig. 2). Another two catabolic enzymes [thiopurine methyltransferase (TPMT) and xanthine oxidase (XO)] compete with and reduce the formation of the active 6-TGNs. XO oxidises 6-MP to thioxanthine (6-TX) and subsequently to thiouric acid (6-TU) whereas TPMT methylates 6-MP to 6-methyl mercaptopurine (6-mMP). In addition, TPMT can methylate TIMP to the methylated derivative, 6-mMP nucleotides (6-mMPNs) which are potent inhibitors of *denovo* purine synthesis [4].

Due to wide inter-individual differences in AZA and 6-MP metabolism among patients receiving identical doses of these agents, monitoring 6-TGNs and 6-mMPNs concentrations in erythrocytes has been proposed as a useful clinical tool for assessing treatment efficacy and toxicity [5–8]. Furthermore, the quantification of 6-TGNs and 6-mMPNs metabolites has been recommended when patients are not achieving therapeutic efficacy despite adequate weight-based dosing to ascertain non-adherence to the prescribed treatment or preferential metabolism away from 6-TGNs favouring 6-mMPNs [9,10].

Despite the extensive clinical experience with 6-MP/AZA, the disposition and metabolism of 6-MP/AZA and their various metabolites remains only partially understood. In addition, sufficient data on the safety and efficacy of AZA when used in Crohn's disease or to prevent rejection in transplantation are lacking, particularly in children. AZA was recently included in the revised "Priority List for Studies into Offpatent Paediatric Medicinal Products" (2008) by the European Medicines Agency [11]. This list is compiled annually under directive from the EU paediatric regulation on Medicinal Products for Paediatric Use (2007) [12] to prioritize off-patent drugs for which paediatric studies are most urgently needed in order to ensure their safety and efficacy.

In the present study, a reversed-phase HPLC method for the rapid simultaneous determination of 6-MP and four of its metabolites;

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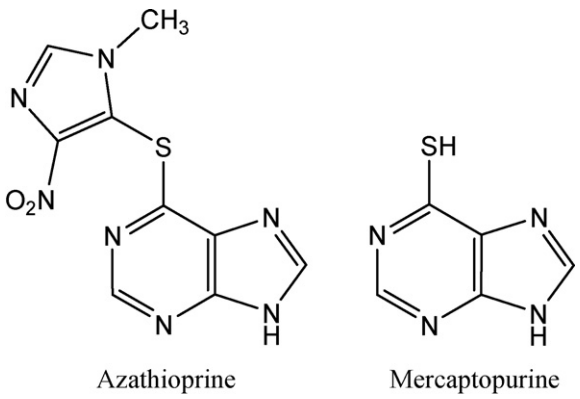

Fig. 1. Structures of azathioprine (1) and mercaptopurine (2).

6-TGNs, 6-TU, 6-TX and 6-mMPNs in both plasma and RBCs was developed and validated in order to facilitate the pharmacokinetic and pharmacogentic assessment of 6-MP and AZA therapy. This method will enable the evaluation of the kinetic nature of the branched tri-enzyme system working on 6-MP/AZA together with investigating the influence of various enzymatic polymorphisms on the different metabolites measured. In addition, the developed method would be used to assess adherence to thiopurine treatment in patients receiving such medication.

Numerous HPLC methods have been developed for the determination of 6-MP and its metabolites in biological fluids [13–16]. In addition, a HPLC-mass spectrometry method for the detection of 6-TGNs and 6-mMPNs has been developed recently [17]. However, the majority of these methods were not suitable for the investigation in the current study as they did not quantify all five compounds of interest. In addition, they were limited by low recovery, laborious and time-consuming sample preparation procedures, the use of neurotoxic reagents, the requirement of multiple extraction procedures together with different sets of chromatographic conditions, time-consuming gradient elution with a total run time reaching 1.5 h in some cases, the requirement of large sample volumes and/or compromised sensitivity. Mass spectrometry methods, on the other hand, are not ideal for routine clinical purposes due to the lack of the availability of the instrumentation in all clinical laboratories. Consequently, this necessitated the development of a method that would overcome these limitations.

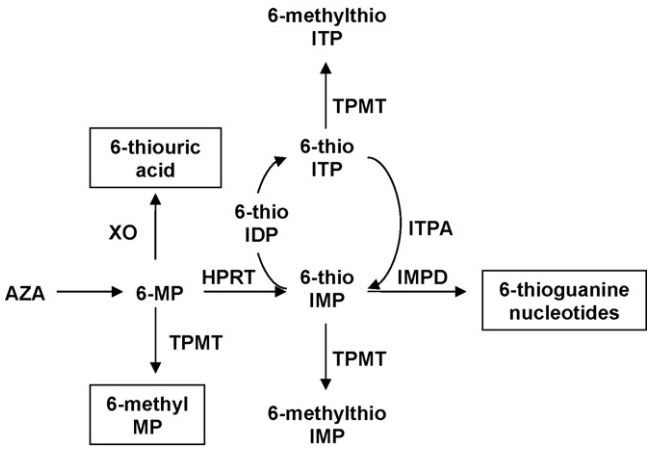

Fig. 2. Thiopurine metabolism. AZA, azathioprine; 6-MP, mercaptopurine; XO, xanthine oxidase; HPRT, hypoxanthine phosphoribosyltransferase; TPMT, thiopurine methyltransferase; IMP, inosine monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; IMPDH, inosine monophosphate dehydrogenase; ITPA, inosine triphosphate pyrophosphatase.

2. Materials and methods

2.1. Reagents and chemicals

All reagents used were of the highest available purity. 6-MP, 6-TG (2-amino-6-mercaptopurine), 6-TX, 6-mMP, D,L-dithiothreitol (DTT), perchloric acid, Hanks' balanced salt solution (HBSS), XO and catalase enzymes were purchased from Sigma (Poole, UK); Potassium dihydrogenphosphate (KH_2PO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH) and trifluoroacetic acid (TFA) were obtained from BDH Laboratories Supplies (Poole, UK); Sephadex LH-20 was purchased from Amersham Biosciences (Uppsala, Sweden); and HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Loughborough, UK).

All water was HPLC grade and prepared in house using a Millipore Direct-Q™ 5 Water System (Millipore, Watford, UK). Filtration of HPLC mobile phases was performed using Sartorius membrane filters [0.45 μm] obtained from Sartorius (Epsom, UK).

Whole blood was kindly donated by the Northern Ireland Blood Transfusion Service and stored at 4 °C until required.

2.2. Thiouric acid synthesis

Because it was not commercially available, 6-TU was synthesized by the enzymatic oxidation of 6-TX by XO in buffer at 37 °C following the method of Bergmann and Ungar [18]. 6-TU formed in the reaction was purified on a Sephadex LH-20 column (Amersham Biosciences, UK) using 5% acetonitrile adjusted to pH 2.5 with TFA. Fractions containing 6-TU were combined and lyophilized. The purity of the final product was greater than 95% as assessed by HPLC.

2.3. Stock solutions

Stock solutions of 6-MP, 6-TG, 6-TX and 6-mMP were prepared by dissolving the appropriate amount of each compound in 0.1 M NaOH and then diluting with water. 6-TU stock solution was prepared by dissolving the appropriate amount of 6-TU in 0.1 M NaOH, diluting with water and then acidifying with 0.1 M HCl. Stock solutions were stored at −20 °C until required.

2.4. Erythrocytes and plasma preparation

EDTA-anticoagulated whole blood was centrifuged at 1000 × *g* for 10 min at 4 °C to separate plasma from erythrocytes. The plasma fraction was stored at −20 °C until analysis. The buffy coat together with the upper layer of erythrocytes was discarded. The remaining erythrocytes were washed twice with HBSS. Cells were finally resuspended in HBSS to yield a density of $\sim 8 \times 10^8$ RBC/200 μl, and the red blood cell count was determined before freezing using an automatic haematologic cell counting device (Sysmex SE-9500 Coulter Counter). The isolated erythrocyte suspensions were stored at −20 °C until required for further processing. Erythrocyte count determined before freezing was used to normalize metabolite concentrations to pmol/8×10^8 erythrocytes.

Whole blood obtained from the blood bank was processed as above and used for the preparation of plasma and erythrocyte calibrators and quality-control samples.

2.5. Sample extraction and hydrolysis

Our extraction procedure was based on Dervieux and Boulieu [16]. 200 μl of plasma or suspended erythrocytes were mixed with 100 μl of DTT (75 mg/ml), 50 μl of water and 50 μl of perchloric acid (700 ml/l for 30 s by vortex mixing in a 1.5 ml Eppendorf tube (Sarstedt, Germany). The tube was then centrifuged for 15 min at 13,000 × *g* at room temperature. The supernatant (300 μl) was

transferred to another Eppendorf tube and heated for 45 min at 100 °C to hydrolyse thiopurine nucleotides into their corresponding bases. During hydrolysis, 6-mMP was converted completely into 4-amino-5-(methylthio)carbonyl imidazole (AMTCI) [16]. The latter product was easily quantified under the same chromatographic conditions used for other bases. After cooling, a 30 μl aliquot was injected onto the column.

2.6. Chromatographic conditions

Chromatographic separation was achieved using reversed phase chromatography with isocratic elution. An Atlantis™ dC_{18} column [150 mm × 3.9 mm (i.d.); particle size, 3 μm; Waters] protected with a guard cartridge [20 mm × 3.9 mm; particle size, 3 μm; Waters] was used as the stationary phase.

The mobile phase, consisting of acetonitrile, methanol and KH_2PO_4 (0.02 M; pH 2.25) (3:1:96, v/v/v) was filtered and degassed through a 0.45 μm filter and pumped at a flow rate of 1 ml/min. The column temperature was maintained at 25 °C.

The HPLC system consisted of a separation module [Alliance 2695; Waters] incorporating solvent degasser and sample HTR COOL, a 2-channel UV/vis detector [Model 2487; Waters] and a column heater [Alliance series; Waters]. The system was controlled using Empower™ software.

UV detection was employed at 322 nm (for the detection of 6-MP and the 6-mMP derivative) and 342 nm (for the detection of 6-TU, 6-TG and 6-TX).

2.7. Preparation of calibrators

Working standard solutions were prepared at seven concentrations for the five different compounds by diluting the stock solutions, as appropriate, using water. The working standards were used to prepare plasma and erythrocyte calibrators by adding 50 μl of spiked aliquots instead of the 50 μl of water during the extraction process. The remaining processing and hydrolysis steps were as described above.

The calibration curves were constructed with 6-TG concentrations of 30–1500 pmol/8 × 10^8 erythrocytes and 10–1250 ng/ml plasma; 6-TU concentrations of 50–1500 pmol/8 × 10^8 erythrocytes and 20–1350 ng/ml plasma; 6-MP concentrations of 10–200 pmol/8 × 10^8 erythrocytes and 10–200 ng/ml plasma; 6-TX concentrations of 10–1500 pmol/8 × 10^8 erythrocytes and 10–1250 ng/ml plasma; and 6-mMP concentrations of 250–24,000 pmol/8 × 10^8 erythrocytes and 100–22,000 ng/ml plasma.[1] These concentrations were chosen to match those predicted in patient samples.

2.8. Assessment of performance characteristics

2.8.1. Selectivity

Possible chromatographic interference was evaluated by separate analysis of blank plasma and erythrocytes samples obtained from untreated healthy subjects (five different sources) and comparing these with plasma and erythrocyte samples spiked with the five different analytes and with plasma and erythrocyte samples obtained from ALL and IBD patients (thirty different sources) treated with 6-MP/AZA therapy. Potential interference from medications commonly administered in patients with ALL or IBD was investigated by analysing samples which had been spiked with the appropriate drug.

[1] By convention, plasma samples were reported in ng/ml and erythrocyte concentrations in pmol/8×10^8 erythrocytes. ng/ml concentrations could be converted to nmol/ml concentrations easily by dividing them by the corresponding MW.

2.8.2. Linearity

The linearity of the method was established by constructing calibration curves for the five analytes, both in plasma and erythrocyte suspensions, on five consecutive days. Plots of peak area (response) against analyte concentration were used. The slope, the intercept and the correlation coefficient of each calibration curve were determined.

2.8.3. Precision and accuracy

Within- and between-day precision and accuracy for the developed method were studied by replicate analyses ($n = 5$) of erythrocyte and plasma samples supplemented with the compounds of interest at four different concentrations representing the very low, low, medium and high portions of the calibration curve. Samples spiked at these concentrations were analysed over five consecutive days and at five replicates of each concentration level during a single day.

The very low, low, medium and high concentrations chosen for 6-TG were 30, 250, 750, 1250 pmol/8 × 10^8 erythrocytes and 10, 150, 650, 950 ng/ml plasma; for 6-TU were 50, 250, 750, 1250 pmol/8 × 10^8 erythrocytes and 20, 150, 650, 950 ng/ml plasma; for 6-MP were 10, 50, 125, 175 pmol/8 × 10^8 erythrocytes and 10, 50, 125, 175 ng/ml plasma; for 6-TX were 10, 250, 750, 1250 pmol/8 × 10^8 erythrocytes and 10, 150, 650, 950 ng/ml plasma; and for 6-mMP were 250, 2500, 7500, 17,500 pmol/8 × 10^8 erythrocytes and 100, 2500, 7500, 17,500 ng/ml plasma.

Precision was expressed as the coefficient of variation (CV%). To obtain information on the accuracy of the method, it was calculated by comparing the measured concentrations with the expected concentrations using the analyte-enriched plasma and erythrocyte quality-control samples described above. Accuracy was expressed as the mean relative error (RE%). A precision (CV%) ≤20% and an accuracy (RE%) ≤20% are acceptable [19].

2.8.4. Recovery

The efficiency of the extraction procedure was determined by the analysis of erythrocyte and plasma samples spiked with the five analytes at the same low, medium and high concentrations used for determining precision and accuracy. Five replicates at each concentration level were extracted and analysed.

The extraction efficiency was calculated by comparing peak areas obtained for the extracted erythrocyte and plasma samples containing the five different compounds with peak areas obtained for aqueous solutions containing the same amount of these compounds which were injected directly onto the column without extraction after incubation at 100 °C for 45 min.

2.8.5. Limit of detection and lower limit of quantification

Limit of detection (LOD) was calculated for each compound based on the standard deviation (σ) of the response and the slope (S) of the calibration curve prepared for each analyte both in plasma and erythrocytes at levels approximating the LOD according to the formula [20]:

$$\mathrm{LOD} = 3.3\,\sigma/S$$

where σ is estimated from the standard deviation of y-intercepts of regression lines.

The lower limit of quantification (LLOQ) was calculated for each compound based again on the standard deviation (σ) of the response and the slope (S) of the calibration curves according to the formula [20]:

$$\mathrm{LLOQ} = 10\,\sigma/S$$

The LLOQ was validated by determining the precision and accuracy with which samples prepared near the LLOQ were quantified.

2.8.6. Stability

The stability of samples during storage was determined by analysing plasma and erythrocyte samples containing 6-MP and its metabolites at two levels ($n = 6$) on two occasions, one month apart, after storage at −20 °C. For each sample, the ratio of the two concentrations measured for each analyte before and after storage, was calculated. The mean ratio and standard deviation for each analyte was then determined.

2.8.7. Statistical methods

Linearity was calculated by linear regression analysis. Standard curve regression analysis was computed by Empower™ software (Waters Corporation, USA) without forcing through zero. Means and standard deviations were calculated using Microsoft® Excel software (Microsoft Corporation, USA).

3. Results

3.1. Chromatographic separation

Representative chromatograms obtained following the sample treatment procedure outlined above, are presented for erythrocytes supplemented with 6-MP and its metabolites (Fig. 3) versus plasma supplemented with these five compounds (Fig. 4). Peak at 12 min is due to DTT used in the extraction step.

The elution conditions used allowed good resolution between 6-MP and its four metabolites in a single run under isocratic conditions both in plasma and erythrocytes. No interfering substances were present in the processed plasma or erythrocytes obtained from untreated healthy subjects or patients with ALL or IBD. In addition, no interference with the commonly co-administered drugs in IBD or ALL patients such as sulfalazine, mesalamine, metronidazole, methotrexate, vincristine, prednisone or dexamethasone was found. Related compounds such as caffeine and theophylline were also separated from the compounds of interest.

The total run time was only 13 min with all peaks of interest being eluted within 7 min. The retention times for 6-TG, 6-TU, 6-MP, 6-TX and 6-mMP were 3.8, 4.3, 4.8, 6.4, 6.9 min in plasma and erythrocyte samples, respectively.

3.2. Deproteinisation step

The sample treatment step is simple, involving deproteinisation using 70% perchloric acid with DTT and hydrolysis of thiopurine nucleotides into their free bases by heating of the acid extract [16]. The final concentration of DTT used in our method was 120 mM.

3.3. Hydrolysis step

A minimum hydrolysis time of 30 min was necessary for complete hydrolysis of 6-TGNs into 6-TG. The hydrolysis time used in our method was 45 min.

The recovery of 6-mMP is greatly influenced by the pH of acid hydrolysis. The conversion of 6-mMP into the derivative during the heating step under the acidic conditions described above is reported to be 100% [16].

3.4. Linearity and precision

The slope, y-intercept and correlation coefficient of the regression line, calculated for each analyte on five consecutive days, are presented in Table 1. The relationship between peak area and analyte concentration was linear over the concentration range studied with a correlation coefficient >0.994 for each compound both in plasma and erythrocytes.

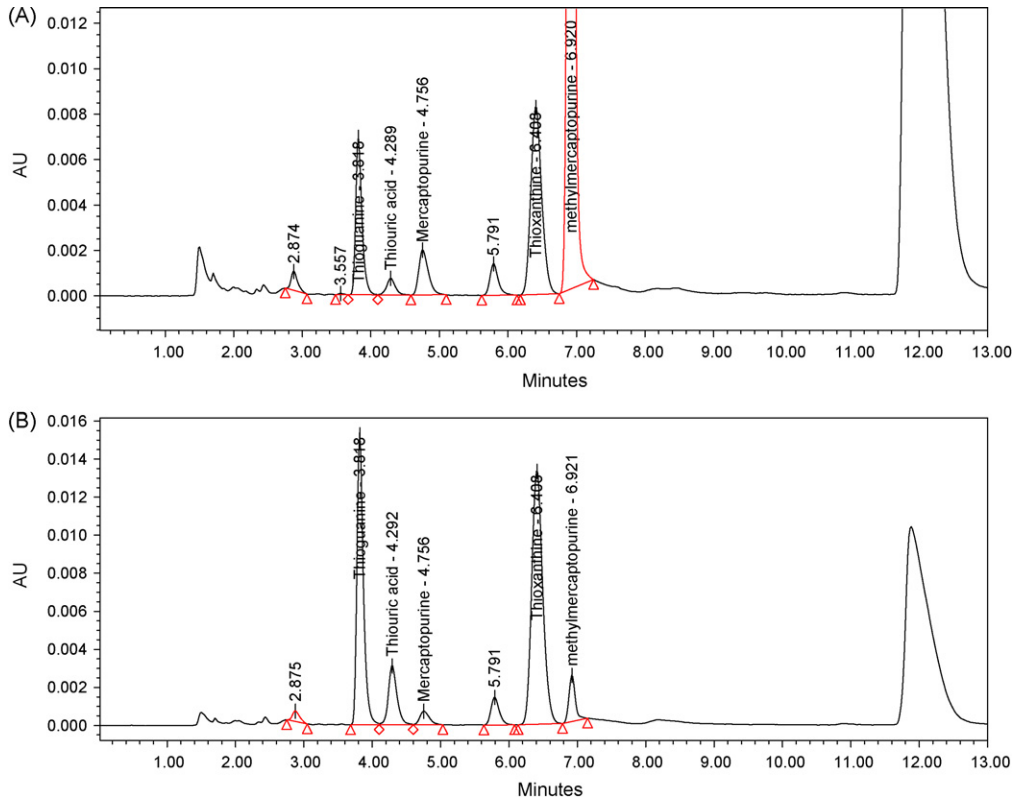

Fig. 3. Chromatograms of erythrocyte sample spiked with 6-TG, 6-TU, 6-MP, 6-TX and 6-mMP at the level of 750, 500, 200, 500, 12,000 pmol/8 × 10^8 erythrocytes, respectively and detected at (A) 322 nm and (B) 342 nm.

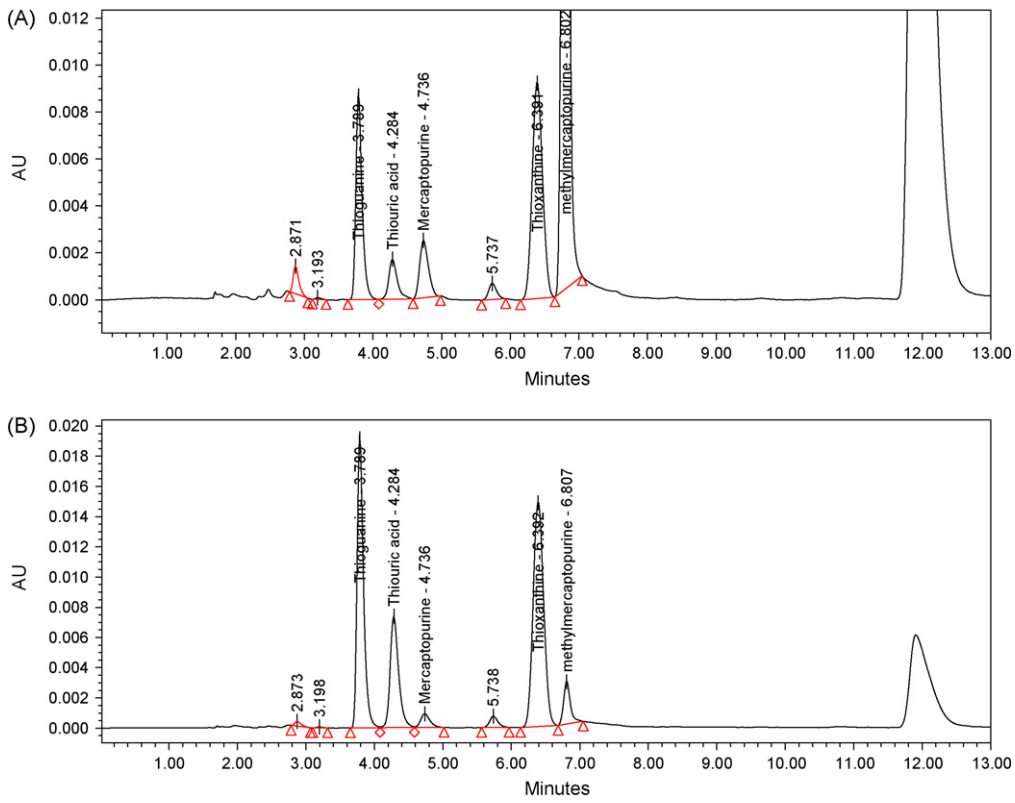

Fig. 4. Chromatograms of plasma sample spiked with 6-TG, 6-TU, 6-MP, 6-TX and 6-mMP at the level of 750, 500, 200, 500, 12,000 ng/ml, respectively and detected at (A) 322 nm and (B) 342 nm.

The intraday and interday precision and accuracy are presented in Tables 2 and 3. All results were within the recommended limits for the five compounds in plasma and erythrocytes. In our method, 6-TU was recovered from erythrocytes with precision and accuracy being within the recommended limits. 6-TU, however, was not recovered from erythrocytes in other methods and zero recovery from erythrocytes has been previously reported [13,21]. The higher 6-TU recovery was probably due to the increased level of DTT used resulting in added protection of thiol group in this compound from oxidation during the sample treatment procedure.

The LOD values for 6-TG, 6-TU, 6-MP, 6-TX, and 6-mMP were determined as 4, 5, 1, 1, 30 pmol/8 × 108 in RBCs and 1, 2, 1, 1, 8 ng/ml in plasma, respectively. The corresponding LLOQ values were determined as 13, 14, 3, 2, 95 pmol/8 × 10^8 RBCs and 2, 5, 2, 3, 20 ng/ml plasma for 6-TG, 6-TU, 6-MP, 6-TX, and 6-mMP, respectively.

The guideline for validation of analytical procedures produced by the International Conference on Harmonisation, recommends that when the LLOQ is determined by calculation, it may be subsequently validated by analysis of a suitable number of samples known to be near the limit [20,22]. The precision and accuracy with which blank plasma and erythrocyte samples spiked with 6-MP and its metabolites near the LLOQ were determined. All results were within the acceptable limits (Table 3).

3.5. Recovery

The efficiency of the extraction procedure for the five analytes in both plasma and erythrocytes, at three concentrations, is shown in Table 4. The mean extraction efficiency was 76.7%, 31.2%, 90.9%, 91.2%, 99.8% for 6-TG, 6-TU, 6-MP, 6-TX, and 6-mMP in erythrocytes and 94.5%, 92.2%, 97.9%, 99.5%, 105.1% for these compounds in plasma, respectively.

3.6. Stability

The mean and standard deviation of the concentration ratios from the six erythrocyte and the six plasma samples analysed were 0.99 ± 0.02, 0.99 ± 0.02, 0.95 ± 0.02, 0.70 ± 0.07, 1.10 ± 0.09 for 6-MP, 6-mMP, 6-TG, 6-TU, 6-TX in erythrocytes and 1.11 ± 0.06, 1.05 ± 0.02, 1.01 ± 0.02, 1.01 ± 0.06, 1.00 ± 0.10 for these compounds in plasma, respectively. All five analytes were therefore stable in plasma and erythrocytes (except for thiouric acid in erythrocytes) for at least one month when stored at −20 °C.

3.7. Analysis of patient samples

The method developed has been utilized for the determination of 6-MP and its metabolites in clinical samples. Twelve clinical sam-

Table 1
Results of the five calibration curves for 6-MP and its metabolites in plasma and erythrocytes: slope ± SD, intercept and correlation coefficient of the standard curves.

Compound name	Erythrocytes			Plasma		
	Slope (mean ± SD)	Intercept (mean)	Correlation coefficient (r)	Slope (mean ± SD)	Intercept (mean)	Correlation coefficient (r)
Thioguanine	71.50 ± 4.03	41.39	>0.999	69.89 ± 2.97	−29.99	>0.999
Thiouric acid	26.44 ± 3.42	−21.183	>0.996	96.52 ± 1.26	27.15	>0.999
Mercaptopurine	88.70 ± 1.32	25.76	>0.999	110.40 ± 2.89	−14.663	>0.994
Thioxanthine	109.84 ± 5.08	−13.11	>0.999	133.44 ± 6.02	−59.92	>0.999
Methylmercaptopurine	14.98 ± 0.53	160.458	>0.999	17.07 ± 0.25	255.54	>0.999

Table 2
The intraday and interday precision and accuracy for 6-MP and its metabolites in erythrocytes ($n = 5$).

Compound	Nominal concentration (pmol/8 × 10^8 erythrocytes)	Intraday ($n = 5$)			Interday ($n = 5$)		
		Precision		Accuracy	Precision		Accuracy
		Mean ± SD	CV%	Mean relative errors (%)	Mean ± SD	CV%	Mean relative errors (%)
Thioguanine	30	28.0 ± 4.7	16.9	−6.8	32.1 ± 5.7	17.6	7.1
	250	248.3 ± 8.7	3.5	−0.7	251.8 ± 7.8	3.1	0.7
	750	760.4 ± 12.0	1.6	1.4	741.9 ± 15.9	2.1	−1.1
	1,250	1,257.3 ± 33.7	2.7	0.6	1,234.4 ± 37.1	3.0	−1.2
Thiouric acid	50	58.4 ± 7.1	12.1	16.8	58.3 ± 7.8	13.4	16.5
	250	250.3 ± 13.3	5.3	0.1	255.3 ± 15.1	5.9	2.1
	750	721.2 ± 22.8	3.2	−3.8	697.5 ± 48.7	7.0	−6.9
	1,250	1,183.0 ± 129.3	10.9	−5.3	1,303.3 ± 80.5	6.2	4.3
Mercaptopurine	10	10.1 ± 1.8	17.4	1.4	10.2 ± 1.6	15.3	2.2
	50	49.5 ± 2.3	4.6	−1.1	50.2 ± 3.0	5.9	0.4
	125	127.7 ± 2.8	2.2	2.2	124.0 ± 3.6	2.9	−0.8
	175	185.9 ± 15.8	8.5	6.3	169.4 ± 8.7	5.1	−3.2
Thioxanthine	10	10.5 ± 1.9	18.1	5.1	10.6 ± 2.0	19.2	5.7
	250	242.8 ± 4.1	1.7	−2.9	250.8 ± 8.0	3.2	0.3
	750	727.5 ± 16.9	2.3	−3.0	737.3 ± 14.2	1.9	−1.7
	1,250	1,255.6 ± 40.2	3.2	0.4	1,252.5 ± 9.7	0.8	0.2
Methylmercaptopurine	250	204.3 ± 39.0	19.1	−18.3	202.5 ± 35.0	17.3	−19.0
	2,500	2,532.5 ± 103.8	4.1	1.3	2,527.5 ± 83.4	3.3	1.1
	7,500	7,552.5 ± 226.6	3.0	0.7	7,590.0 ± 235.3	3.1	1.2
	17,500	17,681.8 ± 760.2	4.3	1.0	17,345.8 ± 521.1	3.0	−0.9

ples from 11 patients with IBD and 34 samples from 19 patients with ALL have been analysed already with metabolite levels being within the ranges quoted in literature [9,13].

Chromatograms of plasma and erythrocyte samples from an IBD patient (female, 68 years old) who received 50 mg of 6-MP per day for 4 months and a child with ALL (male, 5 years old) who received 65 mg of 6-MP per day for 1 month are presented in Figs. 5 and 6. The sample from the IBD patient was taken 3.5 h after the dose, while it was taken on the next day from ALL child. The presence of 6-MP and its metabolites are clearly visible in both patients. The concentrations of 6-TG, 6-TU, 6-MP, and 6-mMP found in erythrocytes were 270.7, 56.2, 9.9 and 1321.3 pmol/8 × 10^8 erythrocytes and 230.0, 161.5, 15.3 and 7078.3 pmol/8 × 10^8 erythrocytes in the IBD and ALL patients, respectively. However, the concentrations of 6-TU and 6-MP in plasma were 129.2 and 45.1 ng/ml in the IBD patient; 6-TU was present at a concentration lower than the LLOQ while 6-MP was not detected in the ALL patient. This is consistent with 6-MP pharmacokinetics since it is not detected in plasma after 8 h or more from dose intake.

4. Discussion and conclusions

In this paper a valid and reliable method for routine analysis of 6-MP and four of its metabolites and which is applicable to

Table 3
The intraday and interday precision and accuracy for 6-MP and its metabolites in plasma ($n = 5$).

Compound	Nominal concentration (ng/ml)	Intraday ($n = 5$)			Interday ($n = 5$)		
		Precision		Accuracy	Precision		Accuracy
		Mean ± SD	CV%	Mean relative errors (%)	Mean ± SD	CV%	Mean relative errors (%)
Thioguanine	10	11.6 ± 1.2	10.2	16.1	11.7 ± 1.3	11.5	16.7
	150	150.0 ± 1.8	1.2	0.0	151.1 ± 2.4	1.6	0.7
	650	650.2 ± 9.5	1.5	0.0	645.9 ± 11.3	1.8	−0.7
	950	949.6 ± 2.2	0.2	0.0	955.0 ± 15.9	1.7	0.6
Thiouric acid	20	21.1 ± 3.5	16.6	5.3	21.2 ± 3.7	17.3	6.2
	150	148.4 ± 3.1	2.1	−1.1	145.4 ± 4.2	2.9	−3.1
	650	652.2 ± 23.1	3.5	0.3	6,41.4 ± 16.7	2.6	−1.3
	950	949.1 ± 13.6	1.4	−0.1	9,57.5 ± 18.5	1.9	0.8
Mercaptopurine	10	9.8 ± 0.9	8.9	−2.1	9.7 ± 0.9	9.1	−3.0
	50	50.3 ± 4.0	7.9	0.5	51.2 ± 1.5	3.0	2.4
	125	124.1 ± 9.9	8.0	−0.8	123.9 ± 5.0	4.0	−0.9
	175	175.5 ± 15.2	8.7	0.3	175.4 ± 7.5	4.3	0.2
Thioxanthine	10	9.8 ± 1.2	12.3	−2.3	9.6 ± 1.4	14.1	−3.7
	150	146.6 ± 1.0	0.7	−2.3	150.2 ± 4.1	2.7	0.1
	650	6,51.4 ± 10.3	1.6	0.2	658.5 ± 8.4	1.3	1.3
	950	9,52.3 ± 13.2	1.4	0.3	956.1 ± 12.3	1.3	0.7
Methylmercaptopurine	100	84.7 ± 10.8	12.7	−15.3	83.2 ± 12.8	15.4	−16.8
	2,500	2,602.5 ± 91.1	3.5	4.1	2,495.0 ± 104.8	4.2	−0.2
	7,500	7,777.5 ± 116.7	1.5	3.7	7,605.0 ± 205.3	2.7	1.4
	17,500	17,674.9 ± 140.7	0.8	1.0	17,538.5 ± 214.2	1.2	0.2

Table 4
The extraction efficiency of 6-MP and its metabolites from both plasma and erythrocytes ($n = 5$).

Compound	Nominal concentration (pmol/8 × 10^8 erythrocytes)	Erythrocytes		Nominal concentration (ng/ml)	Plasma	
		Recovery (%)			Recovery (%)	
		Mean ± SD	CV%		Mean ± SD	CV%
Thioguanine	250	76.9 ± 7.3	9.5	150	94.5 ± 2.7	2.9
	750	76.7 ± 6.0	7.8	650	93.3 ± 2.9	3.1
	1,250	76.4 ± 4.5	5.8	950	95.6 ± 3.0	3.1
Thiouric acid	250	30.9 ± 2.8	9.1	150	91.9 ± 4.1	4.4
	750	29.3 ± 2.3	7.9	650	91.2 ± 2.8	3.0
	1,250	33.5 ± 4.3	13.0	950	93.4 ± 3.2	3.5
Mercaptopurine	50	92.6 ± 5.1	5.5	50	97.6 ± 6.0	6.1
	125	90.2 ± 4.8	5.3	125	96.5 ± 7.5	7.8
	175	89.9 ± 2.8	3.1	175	99.5 ± 10.0	10.0
Thioxanthine	250	92.7 ± 2.2	2.4	150	99.7 ± 5.9	5.9
	750	89.3 ± 0.8	0.9	650	101.0 ± 0.5	0.5
	1,250	91.6 ± 3.0	3.3	950	97.7 ± 2.0	2.1
Methylmercaptopurine	2,500	102.4 ± 3.7	3.6	2,500	106.5 ± 3.9	3.7
	7,500	98.7 ± 3.2	3.2	7,500	105.4 ± 3.3	3.1
	17,500	98.3 ± 0.8	0.8	17,500	103.3 ± 2.4	2.3

both plasma and erythrocytes has been developed. The aim was three-fold; to assess the possible associations of these metabolites with the various polymorphic variations in the genes encoding the three main enzymes involved in 6-MP metabolism, to facilitate the pharmacokinetic modelling of 6-MP/AZA metabolites in different age-subsets particularly children and identify the inter- and intra-individual variability in 6-MP metabolism among the three competing metabolic routes catalysed by HGPRT, XO and TPMT since it permits the quantification of the metabolic end-products of these enzymes. During the present work, a study reported the evaluation of the kinetic nature of the branched bi-enzyme system composed of HGPRT and XO and hence quantified their metabolic products (TIMP and 6-TU) simultaneously [23]. The present method, however, will enable the evaluation of the branched tri-enzyme system working on 6-MP/AZA. In addition to its usefulness in pharmacokinetic and pharmacogenetic assessment of thiopurine therapy, the developed method would also be used to prospectively assess adherence to thiopurine medication in patients receiving such medication. Together with the intra-erythrocyte concentrations of 6-TGNs and 6-mMPNs, the measurement of 6-MP and 6-TU in plasma (indicative of recent drug intake) can be used to monitor adherence to thiopurine medication [24]. Due to the accumulation of the parent drug and its metabolites inside RBC, it is expected that the higher concentration of metabolites within RBC can be used as a better indicator of drug intake than plasma levels.

To our knowledge, there is no HPLC method previously reported for the simultaneous determination of 6-TGNs, 6-MP, 6-TU, 6-TX and 6-mMPNs in a single run and which is applicable to determination in both plasma and erythrocytes. The method described is selective and sensitive enough to analyse 6-TGNs, 6-TU, 6-MP, 6-TX, and 6-mMPNs in a single run under isocratic conditions.

The present extraction procedure was based on that developed by Dervieux and Boulieu [16] because it is rapid, simple and exhibits higher recoveries compared with other methods. Multiple factors seem to be responsible for the higher recoveries achieved by the Dervieux method. Of particular importance are the acid used, DTT concentration and the hydrolysis time chosen. However, some modifications have been implemented in our method regarding the amounts of the different compounds and the centrifugal force used in the extraction step; the volume of erythrocytes used was reduced by more than 50% (200 μl instead of 500 μl), the DTT concentration was doubled (120 mmol/l instead of 60 mM), the centrifugal force increased from 3000 × g to 13,000 × g and the injection volume was reduced from 80 to 30 μl. The increased level of DTT resulted in added protection of the thiol group and contributed to higher 6-TU recovery as compared with previous methods [13,21]. In addition, the presently used chromatographic column (Atlantis^{TM}) contributed to extended column life-time under the acidic conditions used (data not shown).

In the present method, 6-TGNs erythrocyte levels measured were based on the conversion of 6-TGNs to the free 6-TG base. The intracellular 6-TG thus represented the total erythrocyte 6-TGNs contents consisting of a composite of the mono-, di-, and ti-phosphate nucleotides. Likewise, erythrocyte levels of 6-mMP represented the total nucleotides 6-mMPNs rather than the single bases. Since nucleotides are retained in cells due to the presence of a charged phosphate moiety, plasma levels represent on the other hand the free bases which circulate in plasma.

The developed method was found to be selective for 6-MP and its metabolites in the presence of endogenous matrix components. In addition, 6-MP and metabolites were well resolved from the concomitant medications used in ALL and IBD patients. This demonstrated the selectivity of the developed method for all analytes of interest. All results for the intra- and inter-day bias and precision were within the acceptable limits and compared favourably with previously published methods. The lower limit of quantification for 6-TG, 6-TU, 6-MP, 6-TX and 6-mMP were 13, 14, 3, 2, 95 pmol/8 × 10^8 RBCs and 2, 5, 2, 3, 20 ng/ml plasma, respectively and were as good as, or superior to, previously reported assays [13–16].

The main advantage of this HPLC methodology is the rapid simultaneous determination of 6-MP and four of its metabolites. The total run time is only 13 min with all peaks of interest being eluted within 7 min. Furthermore, it allows the measurement of 6-MP and its metabolites using only small amounts of plasma (200 μl) or erythrocytes; such low volume requirements are particularly applicable to low volume paediatric samples. The small volume of blood required together with the simplicity of the analytical technique makes this a useful procedure for monitoring 6-MP cytotoxic

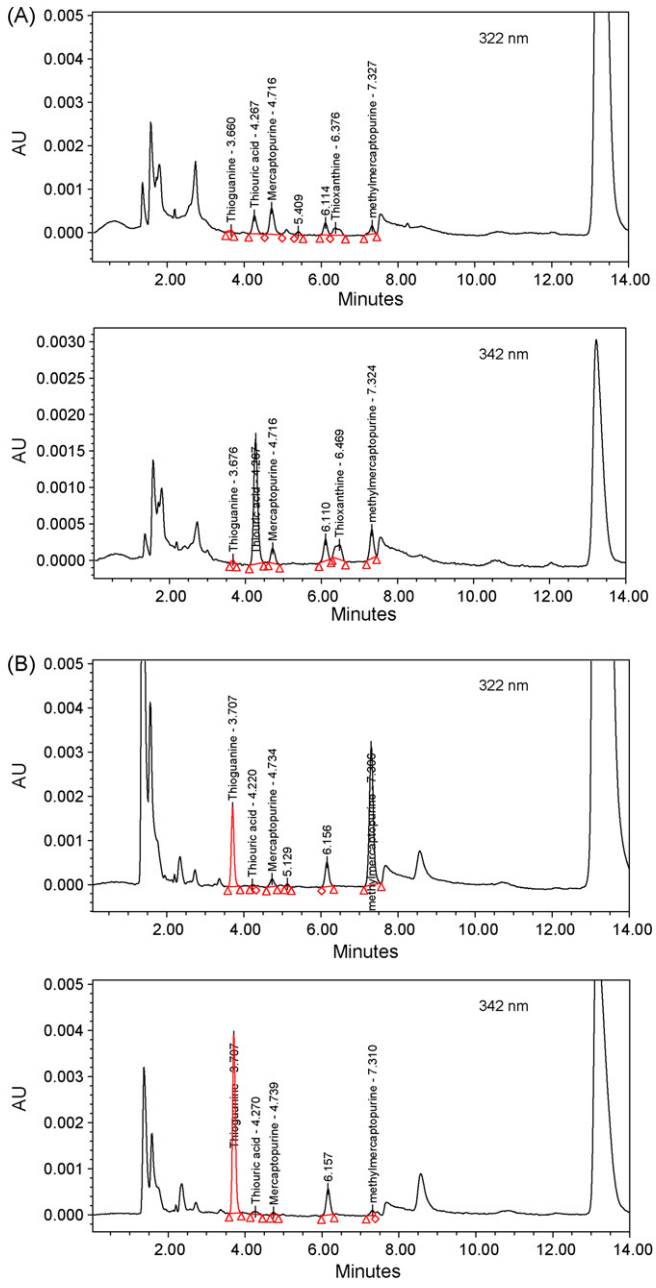

Fig. 5. Chromatograms of an IBD patient's (A) plasma and (B) erythrocyte samples detected at 322 and 342 nm. The IBD patient (female, 68 years old) was receiving a dosage of 50 mg 6-MP for 4 months.

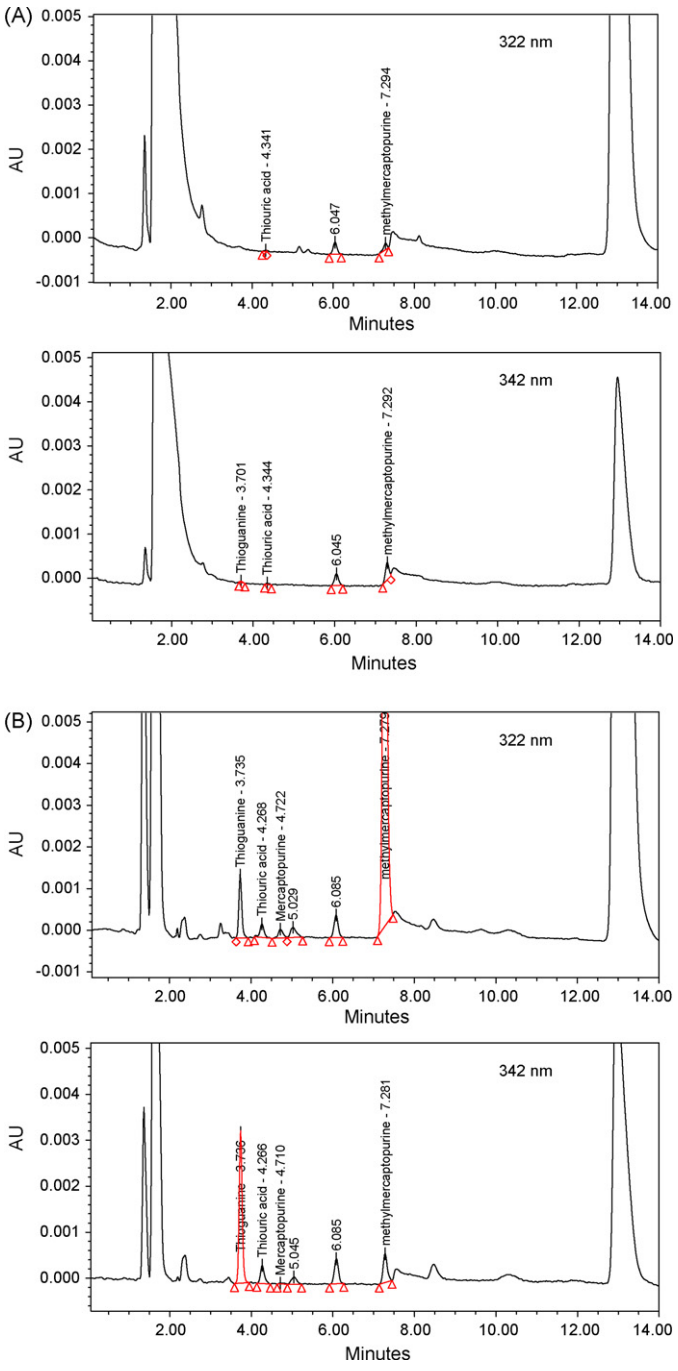

Fig. 6. Chromatograms of an ALL child's (A) plasma and (B) erythrocyte samples detected at 322 and 342 nm. The ALL child (male, 5 years old) was receiving a dosage of 65 mg 6-MP for one month.

metabolites concentrations in routine clinical settings as well as in research studies.

This newly developed method has, as was indicated above, recently been employed in the determination of 6-MP and its metabolites in a study investigating population pharmacokinetics, pharmacogenetics and patient non-adherence to therapy. For this work it was necessary to measure all five components indicated. Although other authors have suggested that the therapeutic outcome for IBD patients can best be determined by chromatographic estimation of 6TGN mono-, di- and tri-phosphate levels this would not be appropriate for the three arms of our study. Furthermore the necessity to individually determine these three compounds has recently been questioned [25].

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