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UPLC–MS/MS method for quantification of the azathioprine metabolites 6-mercaptopguanosine and 6-methylmercaptopurine riboside in peripheral blood mononuclear cells

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Abstract

In the treatment of inflammatory bowel diseases, the use of azathioprine is increasing over the time. It has been demonstrated that the effectiveness of this therapy is modulated by the metabolism of aza- thioprine, which is mainly exerted by both thiopurine methyl-transferase and inosine triphosphatase enzymes. Several studies reported chromatographic methods to determine the amount of its metabo- lites in erythrocytes, but there are not reported methods to dose them in peripheral blood mononuclear cells (PBMCs). The development of a method capable to quantify azathioprine nucleoside metabolites in this compartment could give better information on drug penetration and metabolism in the active site. In this work, we validated a new chromatographic method suitable for the monitoring of the two major biologically active ribonucleos(t)ide metabolites of azathioprine in PBMCs: 6-thioguanosine and 6-methyl- mercaptopurine riboside. After PBMCs extraction from blood through separation on density gradient, samples underwent a de-phosphorylation procedure with acid phosphatase (only one aliquot for each sample) and were then treated with a protein precipitation protocol in acetonitrile, followed by UPLC- tandem-mass spectrometry analysis. The calibration curve for each metabolite in PBMC fitted a least squares model (weighed 1/X) from 0.048 to 25 ng ($r^2 = 0.998$). Both accuracy and precision parameters fitted FDA guidelines. We tested this method by monitoring the concentrations of each metabolite in PBMC from eight inflammatory bowel diseases affected patients, receiving azathioprine maintenance therapy with optimal results.

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1. Introduction

Azathioprine (AZA), a purine analogue, is currently used for the treatment of inflammatory-bowel-diseases (IBD), particularly to handle cases refractory to first-level management. Despite the benefits from this therapy, there is a great inter-individual variability in the effectiveness and in the onset of adverse events related to the treatment, which occurs in up to 20% of treated patients. In particular, the most common problems concerning this therapy are represented by relapses, medullar suppression, hepatic toxicity and pancreatitis [1]. Current studies suggest that differences in the drug metabolism, caused by genetic differences [2,3], are responsible for the different response to the treatment [1,2,4]. Once inside the cell, AZA is converted in 6-mercapto-purine (6-MP) by glutathione- S-transferase and undergoes several metabolic transformations that lead to the production of two different active nucleosides: 6-thioguanosine (6-TG) and 6-methyl-mercaptapurine riboside (6-MMPr) [4]. These metabolites have some differences in their effect on target cells: the current opinion is that 6-TG should be responsible for the immunosuppressive effect, while 6-MMPr should be a secondary metabolism product (dependent by TPMT activity) with a less effective action, but a stronger toxic effect on the liver [1]. So, an overproduction of 6-TG could lead to medullar suppression (excessive effect), instead, an overproduction of 6-MMPr could lead to hepatic toxicity. The decision to undertake the treatment with azathioprine is currently based on the administration of scalar doses of this drug (with an initial dose of 50–100 mg daily), evaluating the clinical response: the decrease of symptoms without toxic effects will lead to the maintenance of the treatment, while the lack of efficacy or the occurrence of severe adverse events will lead to discontinuation of the therapy. High performance liquid chromatography (HPLC) methods were previously developed to quantify azathioprine metabolites levels in erythrocytes, in order to assess the 6-TG/6-MMPr in these cells [5–9]. The common opinion is that the monitoring of these metabolites concentrations could be useful to the treatment personalization [10–12]. However, there is not a concentration cut-off value univocally acknowledged, and the therapeutic drug monitoring is not performed on target cells: lymphocytes and monocytes. To our knowledge, to date there is just one method capable to quantify 6-thioguanosine and 6-methylmercaptapurine riboside only in lymphoblasts [13]. Furthermore, this method was developed for the quantification of these metabolites after intravenous infusion of 6-mercaptapurine (leading to a greater exposure and concentrations), for the treatment of acute lymphoblastic leukaemia. In this study we modified and improved a procedure previously developed for the extraction of another nucleoside analogue (ribavirin) from PBMCs and erythrocytes [14,15] and we developed a new UPLC-MS/MS method for the quantification of these two metabolites. Moreover, the resulting method has been validated according to FDA guidelines [16] and tested for its eligibility for laboratory use by the quantification of eight real-life specimens.

2. Materials and methods

2.1. Chemicals

Acetonitrile HPLC grade and methanol HPLC grade were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Blank PBMC sample were taken from blood of healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy). 6-Thioguanosine, 6-methyl-mercaptapurine riboside, 5*-amino-deoxy-thymidine (5* amino-deoxy-T), dl-dithiotreitol (DTT) and acid phosphatase type 4 from sweet potato were purchased from Sigma-Aldrich Corp (Milan, Italy). The phosphatase enzyme was stored in a refrigerator at 4 °C to prevent degradation. Analytes structures are reported in Fig. 1.

2.2. UPLC instruments and chromatographic conditions

The chromatographic analyses were performed with a Waters Acquity UPLC system, with a binary pump, a refrigerated sample manager and a TQD detector (Waters, Milan, Italy). Chromatographic separation was performed by an Acquity UPLC HSS T3 1.8 μ m, column (150 mm \times 2.1 mm, Waters, Milan, Italy), protected by a pre-column frit (0.2 μ m, 2.1 mm, Waters, Milan, Italy) at 40 °C using the column thermostat. Capillary voltage was set to 1.5 kV. Mass transitions were monitored at 242.3 > 116.1, 300.0 > 168.0 and 299.1 >

167.0 for 5*amino-deoxy-T, 6-TG and 6-MMPr, respectively. Collision energies were 20 eV (5*amino-deoxy-T), 15 eV (6-TG), and 18 eV (6-MMPr), with a cone voltage of 20 V for all molecules. Desolvation temperature was set to 450 °C, with a source temperature of 150 °C. Desolvation gas (nitrogen) was produced by a Parker NitroFlowLab: desolvation and cone flow were 800 and 50 L/h, respectively. Collision gas was Argon. The run was performed with a gradient (Table 1) of two mobile phases: mobile phase A (H₂O with formic acid 0.05%) and mobile phase B (acetonitrile with formic acid 0.05%).

2.3. Stock solutions, standards and quality controls

Stock solutions of 6-TG and 6-MMPr were made in NH₄OH 1 M and methanol, respectively, with a final concentration of 1 mg/mL, and stored at -20 °C until use, no longer than 1 month.

Calibrating solutions were initially prepared at scalar concentration in pure water, and then used, at the time of each analysis, to spike blank aliquots of PBMC (suspended in 500 µL of water:methanol 30:70 v/v), in order to obtain eight standard samples (STDs) and three quality controls (QCs).

The amounts of both compounds in the eight standard samples were, in progress: 0.048 ng, 0.097 ng, 0.194 ng, 0.388 ng, 0.775 ng, 3.125 ng, 6.25 ng, 25 ng. Similarly, three quality controls (QCs), High, Medium and Low were prepared, starting from the same PBMC aliquots. Amounts of 6-TG and 6-MMPr for QC-H, QC-M and QC-L were 20 ng, 0.624 ng and 0.0776 ng, respectively.

Internal Standard (IS) was prepared by diluting 50 µL of a stock solution of 5*amino-5*deoxy-thymidine (1 mg/mL) in 4 mL of a solution of water-methanol 50:50.

STDs, QCs were immediately used, while patients' samples were stored at -80 °C until analyses.

2.4. PBMC preparation procedure

PBMC from patients were extracted from blood (about 12 mL) through a separation on density gradient with two CPT tubes. After the separation (15 min, 1600 × g, 20 °C) the PBMC portion was transferred in a new falcon tube of 50 mL and washed with

40 mL of NaCl 0.9%. After this washing step, supernatant was discarded and pellet was added with 2 mL of ammonium salts solution (ammonium chloride and ammonium carbonate 7 and 0.072 g/L, respectively) and incubated for 1 min at room temperature, in order to eliminate eventually remaining erythrocytes. Then, 38 mL of NaCl 0.9% was added and 500 µL of the resulting cell suspension was transferred in a beaker and put in a Beckman Coulter Z2 (Instrumentation Laboratory, Milan, Italy), managed by Z2 Accu-Comp Software (Version 3.01) for the cell count, as previously described [14,17–19]. This counting method is eligible for the correct determination of the number and volume of PBMCs, evidencing moreover the absence of erythrocytes.

After a new centrifugation, supernatant was discarded and cell pellet was resuspended in 1 mL of H₂O/methanol (30:70 v/v) and frozen at -80 °C.

Blank PBMC from healthy donors were extracted from buffy-coat with a similar procedure: the only difference consisted in the use of a initial separation with Ficoll (Lymphoprep®) as previously described [14,17–19].

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by the analysis of six different blank PBMC samples.

2.6. Enzyme digestion and extraction procedure

Each aliquot of PBMC of 2×10^6 cells from healthy donors was divided into two aliquots (so obtaining two aliquots of 1×10^6 cells) and then spiked with calibrating solutions, in order to obtain STDs and QCs. Then, these aliquots were spiked with 40 µL of IS solution. Real samples underwent the same process, but they were added with 100 µL of pure water, to obtain the same volume of STDs and QCs.

After this step, each sample was centrifuged at $6800 \times g$ for 5 min at 4°C , and the supernatant was transferred in a new PTFE tube; 200 μL of methanol were added to each resulting pellet, then vortexed 5 s and centrifuged again at $20,000 \times g$ for 5 min at 4°C .

Supernatants were added to the corresponding PTFE tube and then dried in vacuum centrifuge at 60°C for approximately 1 h.

Resulting dry pellets were resuspended in 200 μL of a mixture of water, Tris-HCl pH 8 and sodium acetate buffer 1 M pH 4 (in proportions of 10:30:2.5 v/v/v, respectively). Only one aliquot of each sample was spiked with 0.5 units of acid phosphatase, to convert phosphorylated metabolites to the unphosphorylated form, leaving the other one untreated.

So, this procedure resulted in the production of two different calibration curves and two series of QCs: one treated and the other one untreated.

All samples were incubated for 1 h and then added with 20 μL of a solution 0.4 M of DTT, 1 mL of acetonitrile, vortex-mixed 10 s and, finally, centrifuged $20,000 \times g$ for 10 min (without brake) at 4°C to achieve protein precipitation. Supernatants were transferred again in glass shots and dried at 60°C in vacuum centrifuge (around 1.5 h).

Resulting dry extracts were finally dissolved in 100 μL of pure water and 7.5 μL have been injected in the chromatographic system.

2.7. Accuracy, precision, and limit of quantification

Intra-day and inter-day accuracy and precision of the method were determined by assaying in six validation sessions, with double-replicate PBMC samples of all 6-TG/6-MMP_r QCs, treated and untreated. Accuracy was calculated as the mean percent deviation from the nominal concentration. Inter-day and intra-day precisions were expressed as the standard percent deviation at each QC concentration. The calibration curve was based on the eight calibration points described above.

Limit of detection (LOD) in PBMC was defined as the concentration that yields a signal-to-noise ratio of 3. Percent deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be $<20\%$, as requested by FDA guidelines.

2.8. Recovery and matrix effect

Average recovery of 6-TG and 6-MMP_r was determined by comparing the peak height of the extracts from the spiked QC samples (20 ng, 0.624 ng and 0.078 ng) with those obtained by direct injection of the same amount of drug in presence of DTT (the same amount as in the extracted samples). The recovery determination was performed both for treated and untreated samples.

Evaluation of matrix effect was performed comparing peak heights corresponding to unextracted spiked solutions (the same used for recovery determination) with those corresponding to dry extracts of blank PBMC samples, resuspended with the same spiked solutions, according to Taylor et al. [20]. The same procedure was repeated also on extracts from 2 and 4 millions of cells to compare eventual differences accounted by the cell number.

2.9. Stability assessment

The stability of samples stored at -80°C was evaluated comparing the quantified amounts of each metabolite in QC samples at 2, 3 and 4 weeks of conservation, with the same QCs stored at -80°C and freshly prepared (in triple replicate). "Freeze and Thaw" stability was assessed analyzing High and Low QCs after three cycles of thawing and comparing the corresponding amounts.

Stock solution stability was evaluated comparing the peak areas obtained from the direct injection of a diluted solution (50 ng/mL in water) prepared from the stock stored at -20°C for 2 months with another one, at the same theoretical concentration, obtained from a freshly prepared stock.

2.10. Patients recruiting and Intra-PBMC concentration calculation

The method was tested on samples from eight patients, all in maintenance treatment with AZA, after informed consent was given. All patients have been diagnosed with inflammatory bowel disease (Crohn's disease or Ulcerative Colitis) and have been previously genotyped for TPMT rs1800460 and rs1142345 polymorphisms.

Blood sampling at the end of dosing interval (C_{trough}) was performed after written informed consent given.

Once the amounts of 6-TG and 6-MMP_r were obtained in each aliquot, the concentrations were calculated correcting amounts on the basis of PBMC volumes, through the formula: $M/(N^{\circ} \times MCV)$, where M is the amount of each molecule, N^o and MCV were the number and mean cell volume of PBMCs, determined with the cell coulter [15,17–19].

This calculation has been done for both treated and untreated patients' samples.

3. Results

3.1. Sensibility and specificity

A typical chromatogram for the highest amount of 6-TG and 6-MMP_r in phosphatase-digested and undigested PBMC samples is shown in Fig. 2 while IS peaks are reported in Fig. 3. No consistent differences in retention times between treated and untreated samples have been observed. Peaks representing 5'-amino-5-deoxythymidine, 6-TG and 6-MMP_r were observed at retention times of 1.97 ± 0.02 , 3.23 ± 0.01 and 4.75 ± 0.01 min, respectively. No interfering peaks generated from endogenous substances were observed on the chromatograms for "blank" PBMCs. LOQ and LOD were 0.0488 and 0.0244 ng, respectively, for both analytes (Figs. 4 and 5A and B).

3.2. Accuracy, precision, and limit of quantification

Relative standard error for intra- and inter-day assay precision were determined by six different validation sessions. Results of method validation are shown in Table 2.

Calibration curves fitted a least squares model (weighed 1/X) in the range from 0.0488 to 25 ng, with a mean regression coefficient r² of 0.998.

3.3. Recovery and matrix effect

Mean recoveries for 6-TG and 6-MMP_r in treated samples were 107% and 105% respectively, resulting stable among different QC levels (SD 4% and 6%, respectively).

In untreated samples, mean recoveries changed to 101% and 77% for 6-TG and 6-MMP_r, respectively (SD among different QCs 14% and 6%, respectively). Mean recovery for IS was 98% with SD of 5% in treated samples, and 93% with SD of 7% in untreated samples.

Mean matrix effect in treated samples was +10% and +14% (SD among different QCs 8% and 5%) for 6-TG and 6-MMP_r, respectively; in untreated samples, matrix effect was -7% and -9% (SD among different QCs 14% and 5%) for 6-TG and 6-MMP_r, respectively.

Matrix effect did not result influenced by cell number: the mean difference in signals obtained in extracts from aliquots of 2 and 4 million cells, compared with the standard 2 millions cells aliquots, was only 2 and 3%, respectively.

3.4. Stability

Stock solution did not show a degradation after 1 month of storage at -20 °C (+3% deviation from freshly prepared stock solution). "Freeze and Thaw" and -80 °C stability data observed at High and Low QC levels are fully reported in Table 3. 6-TG and 6-MMP_r resulted stable at -80 °C for 5 weeks and were not consistently degraded for up to three freezing and thawing cycles.

3.5. Analysis of samples from patients

The median number of extracted PBMCs and MCV were 1,108,380 (inter-quartile range, IQR. 419,650–1,954,330) and 305 fL (IQR 292–322 fL). Median concentrations of total 6-TG and 6-MMPr were 375 ng/mL (IQR 212–1357 ng/mL) and 709 ng/mL (IQR 95–1520 ng/mL), respectively. Only one sample had undetectable 6-MMPr intracellular concentration. The un-phosphorylated fraction of 6-TG and 6-MMPr was under the limit of quantification in 4 out of 8 and 6 out of 8 samples, respectively, evidencing that azathioprine metabolites are present mainly as phosphorylated compounds (nearly 10 folds higher than un-phosphorylated form).

Seven out of eight patients had homozygous wild-type (*1/*1) genotype, only one patient carried a *3A haplotype (rs1800460 G>A and rs1142345 A>G): the median ratio between total 6-TG and 6-MMPr ([6-TG]/[6-MMPr]) was 0.50 (IQR 0.18–0.53) in wild-type homozygotes, while it was 2.34 in the only heterozygote patient.

4. Discussion

In the treatment of inflammatory bowel diseases, the use of azathioprine is increasing over the time. It has been demonstrated that the effectiveness of this therapy is modulated by the metabolism of azathioprine, mainly by TPMT and ITPA enzymes; the evaluation of concentrations in biologic fluid of the biologically active metabolites could help clinicians in the therapy modulation. Several methods have been developed to determine the amount of 6-TG and 6-MMPr in erythrocytes, but up to now there were not methods to dose them in PBMCs.

Our method is the first capable to successfully quantify the total concentration of 6-TG and 6-MMPr nucleotides and nucleosides in PBMCs from IBD affected patients, treated with AZA.

As a whole, six validation tests were performed and, as reported, the values of precision and accuracy obtained for the quality controls during the validation sessions fit perfectly within the range of 15% imposed by the FDA guidelines. The value of LOQ allows a precise and accurate dosage of the total intracellular concentrations of 6-TG and 6-MMPr for the majority of the real PBMC samples extracted with our method. With our method we observed a difference of more than 10 times between the total and the un-phosphorylated fraction of the two metabolites, which was often under the LOQ. The evidence of a strong phosphorylation of 6-TG and 6-MMPr at the active site has never been previously reported.

However, the very low amount of nucleoside forms of 6-TG and 6-MMPr suggests to use their determination only as a good control for the treatment with acid phosphatase.

Our method successfully identified the only one patient with a low activity variant on TPMT gene, evidencing in this patient an extremely higher ratio between intracellular total 6-TG and 6-MMPr as compared with wild-type homozygotes. This is very important because it confirms evidences observed in red blood cells and suggests that the monitoring of metabolites intracellular concentrations (both intra-PBMC and intra-RBC ones) is concordant with the genetic information.

However, further studies are needed to evaluate if this tool offers more useful information than the only genetic or metabolic screening of patients [21].

Each aliquot of extracted PBMC corresponds to only 6 mL of blood from patients in maintenance therapy with AZA: the immunosuppression of these patients could explain the low mean number of PBMC for aliquot.

The use of an automatic cell coulter resulted important for the correct simultaneous determination of the MCV and number of PBMCs, preventing issues due to inaccuracy in the cell count and to the adoption of an approximate MCV. So, using these data for the calculation of intracellular concentration, we adjusted the concentrations of 6-TG and 6-MMPr to the real cellular composition of PBMC samples.

An interesting observed issue was the need of DTT also in unextracted samples (water spiked with metabolites) to detect the right amount of 6-TG. The absence of this issue for 6-MMPr makes us to suppose that the phenomenon could be due to the free-to-react thiolic group in the 6-TG molecule (although no peak corresponding to by-products has been identified by full MS scan).

Our method is based on an enzyme dependent de-phosphorylation, as that used by Dervieux et al. [13] and which we use in other validated methods for the quantification of phosphorylated ribavirin [14,15]. This procedure replaces the acid hydrolysis and the use of heat, which are required for the conversion of nucleosides to free bases [8,9,22].

The other main important difference consists in the capability of this method to discriminate the nucleoside (in untreated samples, very low) from the nucleotide form (much higher, in treated samples) of both main azathioprine metabolites. However, we cannot determinate the different amounts of mono-, di- and tri-phosphate forms of nucleotides.

The large recovery percentages, around 100% for both 6-TG and 6-MMPr in treated samples and for 77% for 6-MMPr only in untreated samples, makes us confident on a good extraction yield and on the short term stability of the compounds at our working conditions. Also a low matrix effect was observed: it was positive in treated samples (+10% and +14% for 6-TG and 6-MMPr, respectively) and negative in untreated samples (-7% and -9% for 6-TG and 6-MMPr, respectively), but within limits requested by FDA. Moreover, this effect seemed to be quite stable (the maximum standard deviation was 14% for 6-TG in untreated samples) between different concentrations and not influenced by cells number. Furthermore, the chosen number of PBMC for standard and QCs was comparable to that in real specimens.

Finally, in a recent work from our group [14], we observed the same phenomenon in RBV intracellular determination, and it was proved to be only related to the use of buffered solutions (the pH 4 sodium acetate buffer and the solution in which acid phosphatase is conserved).

Both metabolites resulted stable at our storing conditions for at least 5 weeks at -80 °C and resistant up to three freezing and thawing cycles.

Moreover, the advantages of our method, respect to others, are: the fast run (7 min); the use of an enzymatic dephosphorylation, avoiding the use of strong acids and high temperature; the cheap extraction method through protein precipitation and the adoption of a cheap internal standard.

Finally, the use of a double calibration curve (treated and untreated), although it is cumbersome, grants a higher reliability of results for both treated and untreated samples.

Possible limit consists in the two drying steps, making the extraction procedure time consuming.

5. Conclusions

In conclusion, this UPLC-MS/MS method results suitable for the simple, accurate and precise dosage of PBMC concentrations of total 6-TG and 6-MMPr. Nowadays, no published studies have investigated the correlation between 6-TG and 6-MMPr concentrations in PBMC and response/toxicity. The only markers already used for therapeutic drug monitoring are the concentrations of these metabolites in erythrocytes: however, the determination of the real number and volume of erythrocytes used for the analysis is difficult and the measurement in erythrocytes is only a surrogate of the quantification of the active metabolites in the target cells.

For these reasons, this method could give a new tool to the clinician to obtain the most useful information upon the concentration of the AZA major metabolites at the active site, allowing them to prevent relapses and toxic effects.

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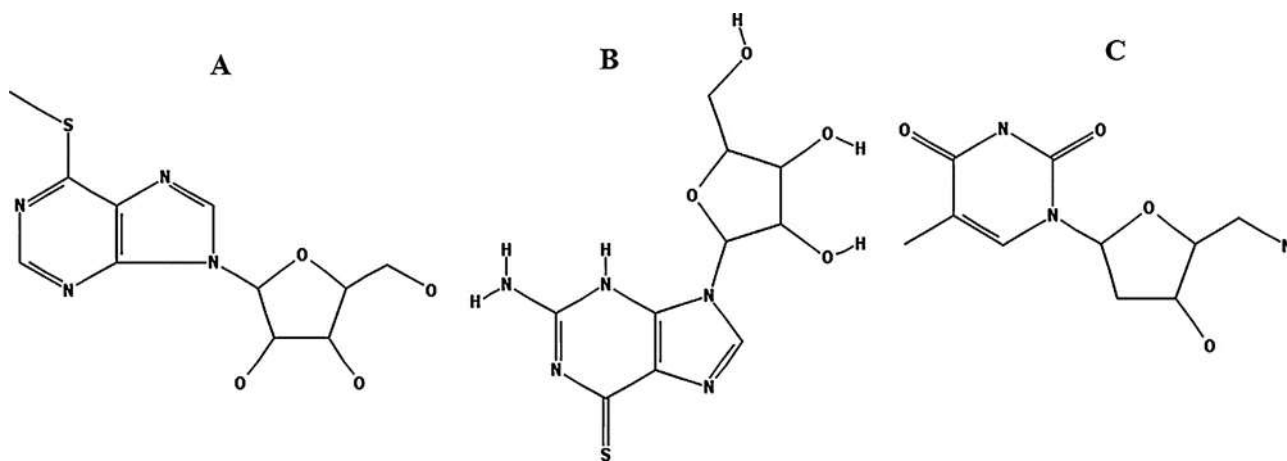


Fig. 1. Chemical structures of target analytes: 6-MMPr (A), 6-TG (B) and 5-amino-5-deoxy-thymidine (C).

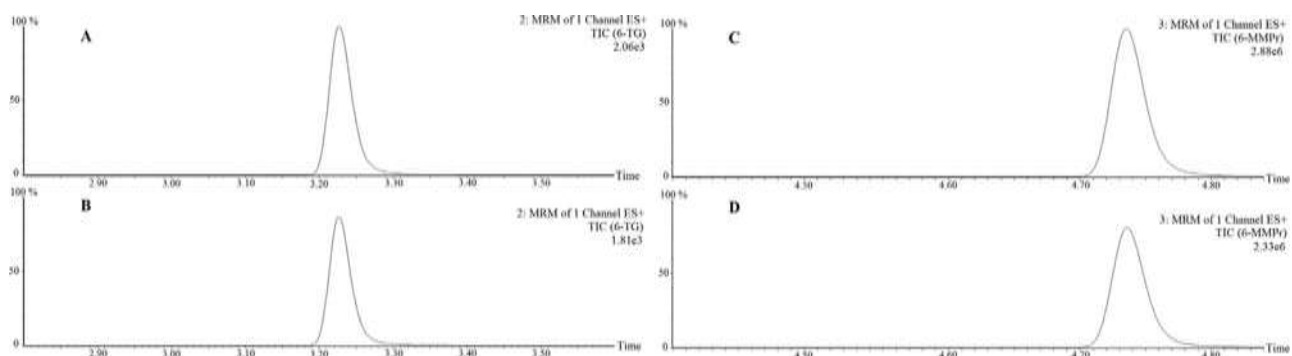


Fig. 2. Chromatograms corresponding to the highest standard point of the calibration curve: Parts (A) and (B) show the signal of 6-thioguanosine and in treated and untreated samples, respectively, and (C) and (D) that of 6-methyl-mercaptopurine riboside in the same samples.

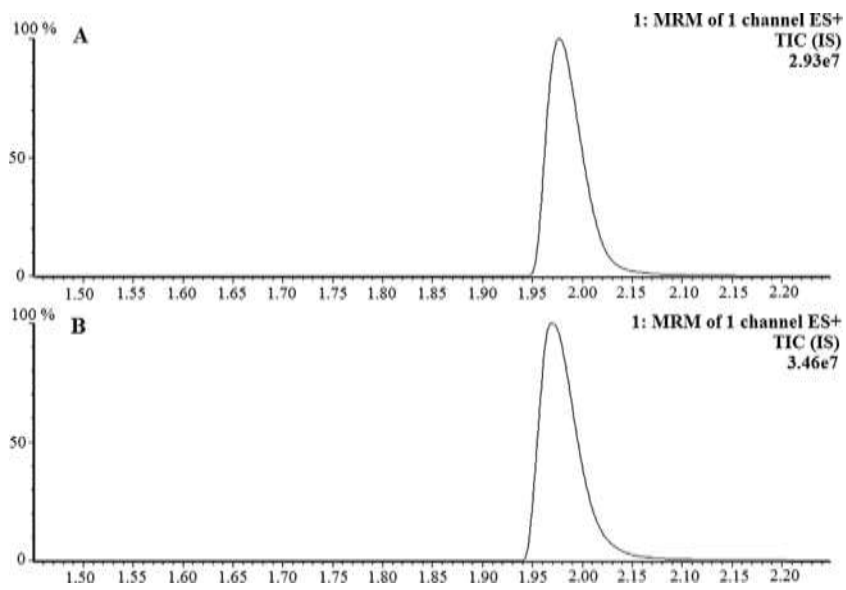


Fig. 3. Chromatographic peaks of 5*-amino-5*deoxy-thymidine in treated (A) and un-treated (B) samples.

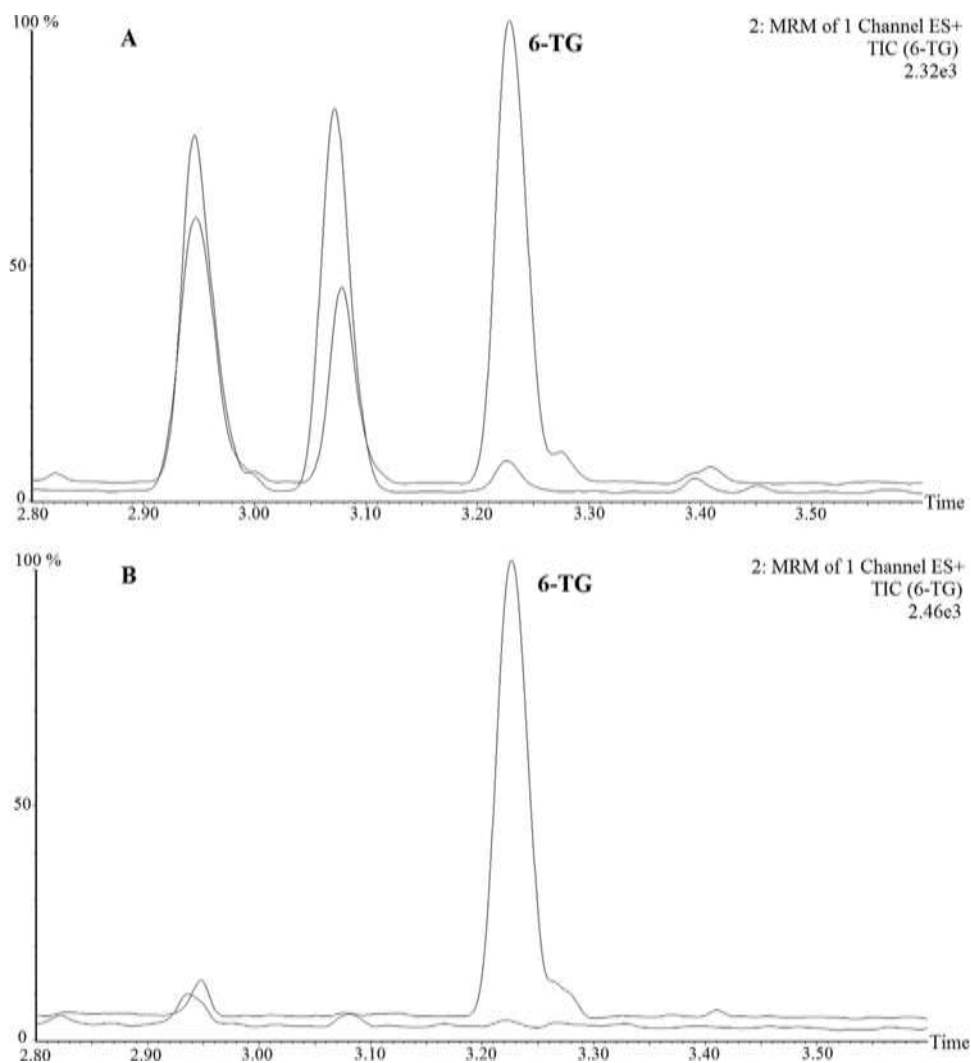


Fig. 4. Comparison between chromatographic peaks of 6-thioguanosine at the limit of quantification (upper line) and in the blank extracts (bottom line) if treated (A) or untreated (B).

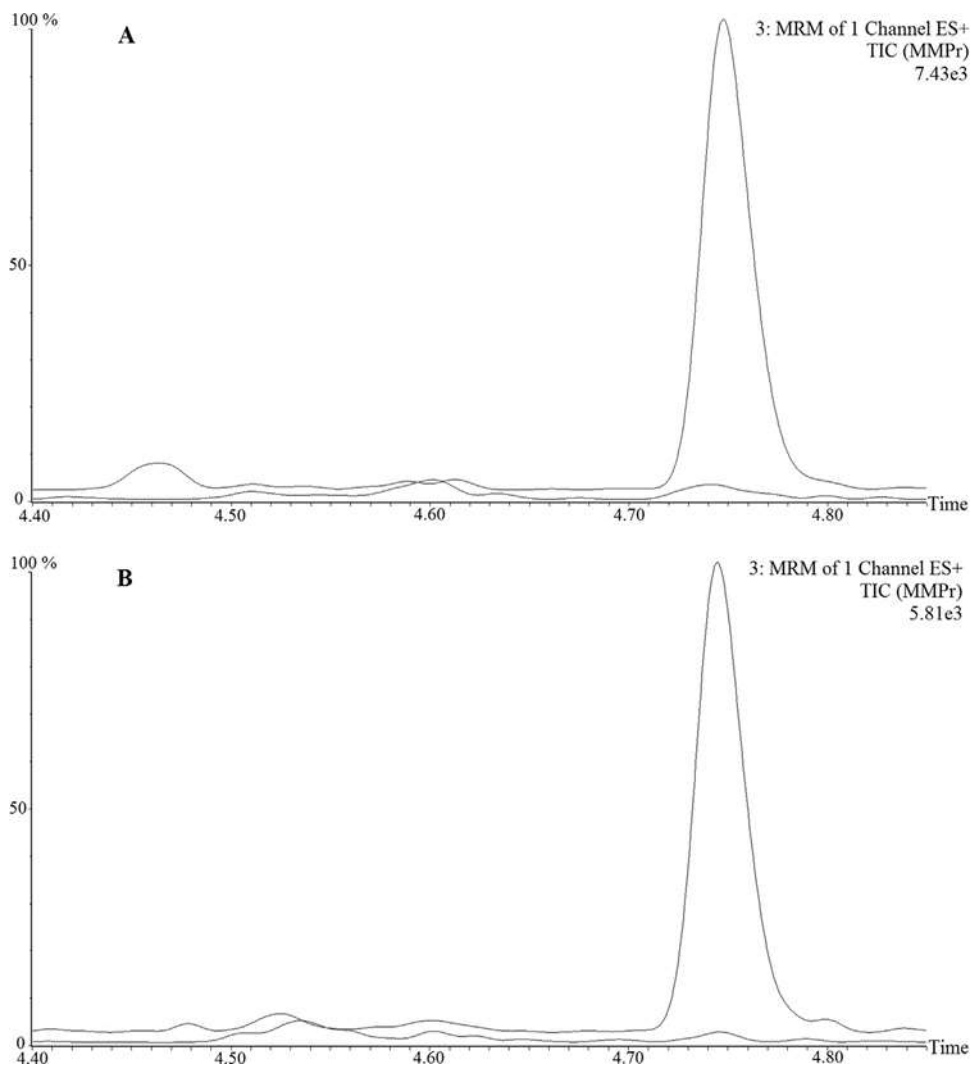


Fig. 5. Comparison between chromatographic peaks of 6-methyl-mercaptopurine riboside, at the limit of quantification (upper line) and in the blank extracts (bottom line) if treated (A) or untreated (B).

Table 1

Chromatographic conditions (gradient). Mobile phase A (H₂O + 0.05% formic acid) and mobile phase B (acetonitrile + 0.05% formic acid).

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.400	99.0	1.0
0.10	0.400	99.0	1.0
1.10	0.400	97.0	3.0
2.40	0.400	92.0	8.0
4.10	0.400	70.0	30.0
4.50	0.400	95.0	5.0
5.50	0.400	95.0	5.0
5.60	0.400	99.0	1.0
7.00	0.400	99.0	1.0

Table 2

Overview of intra- and interday imprecision and accuracy of the assay for the quantification of 6-thioguanosine and 6-methylmercaptopurine-riboside in PBMC extracts: all values fit the acceptance criteria by FDA.

	Intraday imprecision (treated-untreated)	Interday imprecision (treated-untreated)	Accuracy (treated-untreated)
H 6-TG	4.27-3.01%	4.50-3.87%	101.21-105.56%
M 6-TG	5.14-4.17%	8.56-8.40%	95.85-98.00%
L 6-TG	4.21-5.09%	14.42-6.01%	94.10-101.67%
H 6-MMI	4.78-4.16%	6.32-4.29%	92.99-101.19%
M 6-MMI	4.32-3.40%	9.79-4.5%	98.22-100.20%
L 6-MMI	5.20-2.95%	11.35-6.45%	93.70-90.81%

Table 3

Overview of the analytes stability at -80 °C. Stability data are referred to a comparison with fresh-prepared QCs and RSD (%) are referred to triple replicate data from high and low QCs levels.

	Stability at -80 °C			
	Treated QCs		Untreated QCs	
	6-TG % (RSD %)	6-MMPr % (RSD)	6-TG % (RSD)	6-MMPr % (RSD)
2 weeks	100.6 (6.5)	101.3 (10.3)	108.9 (0.4)	100.7 (7.8)
3 weeks	103.6 (6.6)	103.9 (4.3)	104.3 (10.2)	107.7 (4.4)
5 weeks	108.4 (6.9)	104.6 (6.4)	97.8 (4.1)	100.9 (1.8)
One freezing and thawing cycle	101.1 (6.0)	100.9 (8.9)	101.2 (5.1)	101.3 (9.2)
Two freezing and thawing cycles	99.3 (6.9)	86.1 (9.0)	99.2 (6.8)	91.5 (4.1)
Three freezing and thawing cycles	95.5 (2.6)	99.3 (12)	99.6 (8.4)	90.1 (4.1)