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## *Highlights*

- New HILIC-HPLC-MS method was developed for determination of TPMT activity
- HILIC-HPLC-MS combination was approved by excellent performance parameters
- HILIC-HPLC-MS was compared to traditional RP-HPLC-UV as well as advanced RP-HPLC-MS
- Conventional approaches were surpassed in terms of selectivity, LOD, analysis time
- HILIC-HPLC-MS is favorable for routine assay of 6-MMP/6-MP ratio in RBC lysates

# Determination of thiopurine *S*-methyltransferase activity by hydrophilic interaction liquid chromatography hyphenated with mass spectrometry

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## List of abbreviations

**6-MMP** – 6-methylmercaptopurine, **6-MP** – 6-mercaptopurine, **6-TG** – 6-thioguanine, **AZA** – azathioprine, **Cl-P** – 6-chloropurine, **DTT** – dithiothreitol, **IBD** – inflammatory bowel diseases, **RBC** – red blood cells, **SAM** - S-adenosyl-L-methionine, **TGN** – thioguanine nucleotides, **TPMT** - thiopurine-S-methyl transferase.

## Abstract

Thiopurine *S*-methyltransferase (TPMT) plays an important role in the metabolism of thiopurines used in the therapy of inflammatory bowel diseases (IBD). In this work a new progressive method for the determination of TPMT activity in red blood cells lysates was developed. Analysis was carried out by means of hydrophilic interaction liquid chromatography (HILIC) hyphenated with mass spectrometry (MS). In comparison with reversed-phase high-performance liquid chromatography (RP-HPLC), that has been typically applied in determination of TPMT activity, the HILIC significantly improved the analytical signal provided by MS, shortened analysis time, and improved chromatographic resolution. The HILIC-HPLC-MS method was optimized and validated, providing favorable parameters of detection and quantitation limits (5.5 and 16.5 pmol/mL, respectively), linearity (coefficient of determination 0.9999 in the range of 0.01-1.0 nmol/mL), recovery and precision (93.25-100.37% with RSD 1.06-1.32% in the whole concentration range of QC samples). Moreover, in contrast to the conventional RP-HPLC-UV approach, the complex phenotype TPMT profiles can be reliably and without interferences monitored using the HILIC-HPLC-MS method. Such advanced monitoring can provide valuable detail information on the thiopurines (e.g. evaluating ratio of methylated and non-methylated 6-mercaptopurine) and, by that, TPMT action in biological systems before and during the therapy of IBD.

**Key words:** HILIC-HPLC-Q-TOF MS analysis; inflammatory bowel diseases; red blood cell lysates; thiopurine *S*-methyltransferase activity determination.

## 1. Indroduction

Thiopurine drugs, namely azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are immunosuppressive agents that are, due to their low cost and high effectiveness, widely used in the treatment of inflammatory bowel diseases (IBD), but also in other autoimmune diseases, some hemopoetic disorders and some solid organ transplant [1–5]. However, the occurrence of adverse drug reactions limits the use of these drugs. Up to 15 % of IBD patients discontinue this type of treatment due to adverse events that include nausea, skin reactions, pancreatitis, hepatotoxicity or myelotoxicity [6,7].

Thiopurine drugs are prodrugs, so their biological activity is preceded by extensive metabolism. Briefly, after absorption, AZA is metabolized in the liver to 6-MP which then can be metabolized to active thioguanine nucleotides (TGN) and to inactive methylated product 6-methylmercaptopurine (6-MMP) by the thiopurine-S-methyl transferase (TPMT) enzyme [8]. TPMT (EC 2.1.1.67), a key enzyme involved in the thiopurine drug metabolism, is subject to common genetic polymorphism that leads to trimodial distribution of the TPMT activity in population [9]. Individuals with lower TPMT activity might have an increased risk of developing thiopurine-induced myelosuppression. On the contrary, individuals with high TPMT activity have lower concentrations of active TGN metabolites resulting in reduced therapeutic efficacy of the drug [10]. In addition, high TPMT activity leads to accumulation of the hepatotoxic methylated metabolites [11]. Therefore, some scientific committees advise to determine the TPMT activity before the start of thiopurine treatment [12] in order to predict myelo- and hepatotoxicity and adjust the dose accordingly.

The evaluation of TPMT activity is based on the determination of the actual enzymatic activity in red blood cells (RBC) by measuring amount of 6-MMP or 6-methylthioguanine (6-MTG) produced by the TPMT-mediated methylation of 6-MP or 6-TG in presence of the methyl donor, most commonly S-adenosyl-L-methionine (SAM) [13,14].

The very first analytical method used for determination of the methylated products produced by TPMT was radiochemical assay published by Weinshilboum et al. [15]. In this method S-adenosyl-L-[$^{14}$C] methionine was used as the methyl donor and the products created during incubation were detected by liquid scintillation counting. Although this analytical approach was preferred for many years it is associated with several disadvantages such as work with radioactive material, complexity of analytical protocol, requirements on liquid-liquid or solid-phase extraction. Moreover, the radiochemical method is more time-consuming than currently preferred HPLC methods [16]. Over the years more suitable methods for TPMT phenotyping were developed, namely mass spectrometry [14], capillary electrophoresis combined with UV detection [17] or, most commonly, HPLC coupled with UV/DAD [16,18–27] or fluorescence detection [28]. Although separations with non-selective UV detection have been preferred due to their simplicity and low cost, increasing demands on the reliability of data and more detail sample profiles stimulated development of more advanced methods. In this area, only one method employing HPLC hyphenated with MS detection has been published so far [29]. However, this HPLC-MS method was not validated and only limited number of data is available thus far. In summary, the methods currently used for TPMT phenotyping are relatively time-consuming and/or poorly validated which precludes them from wide-spread use in optimizing the treatment with thiopurines.

The aim of this work was thus to develop a new method for TPMT phenotype. For this purpose, hydrophilic interaction liquid chromatography (HILIC) hyphenated with mass spectrometry (MS) was tested as a progressive alternative to the reversed-phase (RP)-HPLC methods that have been used for the determination of the TPMT activity so far. In order to see progress and effectivity enhancement of the proposed HILIC-HPLC-MS method, it was compared with traditional (RP-HPLC-UV) as well as newer (RP-HPLC-MS) approaches through their performance parameters, analysis time, and information value of the recorded sample profiles.

## 2. Materials and methods

### 2.1 Chemicals and solutions

6-MP, 6-MMP, SAM and 6-chloropurine (Cl-P) were obtained from Sigma-Aldrich (Steinheim, Germany). Dithiothreitol (DTT) was obtained from AppliChem GmbH (Darmstadt, Germany). Other chemicals were obtained from Fluka Chemie GmbH (Buchs, Switzerland). All chemicals used were of analytical grade. All solutions were prepared in water demineralized by a Direct-Q 3 UV water purification system (Merck Millipore, Molsheim, France).

Phosphate buffer solution (50 µmol/mL) was prepared by mixing equimolar amounts of $Na_2HPO_4$ and $NaH_2PO_4$ and adjusting the pH to 7.4 with NaOH. The stock solutions of 6-MP (30 µmol/mL) and 6-MMP (0.1 µmol/mL) were prepared in 100 µmol/mL NaOH. The stock solutions of SAM (6 µmol/mL) and DTT (50 µmol/mL) were prepared by dissolving the standards in phosphate buffer solution. The stock solution of Cl-P (0.1 µmol/mL), serving as an internal standard, was also prepared by dissolution in the phosphate buffer solution. All of the stock solutions were kept frozen at - 20 °C until use.

### 2.2. Procedures for sample and standard solution preparations

### 2.2.1 Standard solutions

The calibration solutions of 6-MMP were prepared in 100 µmol/mL NaOH and in the matrix, which was prepared in same manner as the RBC samples but the addition of 6-MP was omitted. The calibration solutions used in the RP-HPLC-DAD, RP-HPLC-MS, and

HILIC-HPLC-MS methods were prepared in the concentration ranges of 0.1 – 10.0, 0.1 – 5.0, and 0.01 – 1.00 nmol/mL, respectively. Each calibration point was measured in duplicate.

The QC samples were prepared by spiking the precipitated RBC lysate (matrix) with the stock solution of 6-MMP. The QC samples at three concentration levels (low, medium, high, 0.1, 0.5, 1 nmol/mL, respectively) were prepared, and each was measured five times.

### 2.2.2 Blood sample collection and preparation

Blood samples from healthy volunteers were collected in EDTA tubes and immediately placed into the fridge at 4 °C where they were stored until further processing. All the samples were processed within 24 h of the blood sampling.

RBC were separated from the whole blood by the centrifugation at 800 *g* for 10 min at 4 °C. After the centrifugation, the plasma, buffy coat and upper layer of RBC were discarded. The isolated RBC were washed by the resuspendation in a 0.9 % saline solution followed by the centrifugation at 800 *g* for 5 min at 4 °C, and, finally, by the removal of the saline wash solution. The washing step was repeated twice. After the second washing step, the packed and washed RBC were diluted with a 0.9 % saline solution (1:1, v/v). Afterwards, the diluted RBC were frozen and stored at - 20 °C until incubation and analysis.

### 2.2.3 Incubation conditions

The determination of TPMT activity is based on the reaction of the substrate (6-MP) with the methyl donor (SAM) that is catalyzed by the TPMT enzyme and results in the methylated product (6-MMP). The reaction mixture (final volume 500 μL) consisted of 325 μL of phosphate buffer solution, 10 μL of 6-MP stock solution (final concentration 600 nmol/mL), 10 μL of 0.1 mmol/mL HCl, 10 μL of SAM stock solution (final concentration 120 nmol/mL), 10 μL of DTT stock solution (serving as inhibitor of oxidation of the thiols; final concentration 1 μmol/mL), and 10 μL of Cl-P stock solution (internal standard). The mixture was shaken and the enzymatic reaction was started by the addition of 100 μL of a freshly thawed RBC lysate under a 37 °C incubation. After 1 hour the enzymatic reaction was stopped by the addition of 25 μL of perchloric acid (60 %) to the reaction mixture which resulted in precipitation of the sample. The precipitated sample was centrifuged at 12500 *g* for 10 min. The supernatant was filtered through a 0.22 μm nylon syringe filter (AZ Chrom, Bratislava, Slovakia), and used for the HPLC analysis. In case of the HILIC column, the supernatant was diluted 10 times in the initial mobile phase.

### 2.3 Apparatus

All of the analyses were performed on the chromatographic apparatus consisting of LC Agilent Infinity System equipped with a gradient pump (1290 Bin Pump VL), an automatic injector (1260 HiPals), and column thermostat (1290 TCC), coupled with photodiode array detector (Infinity 1290 DAD), and quadrupole time-of-flight mass spectrometer (6520 Accurate Mass Q-TOF LC/MS) equipped with electrospray ionization source operated in positive ionization mode, and a computer with Mass Hunter software (version MassHunter Workstation B.05.01) for data acquisition and processing (Agilent Technologies, Santa Clara, CA, USA). Parameters of calibration line for 6-MMP were calculated by using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). The thermostat of the autosampler was set at 4 °C. The 6-MMP was evaluated at the wavelength of 280 nm. The following parameters were used for the mass spectrometer: drying gas temperature 300 °C, drying gas flow 10 L/min., nebulizing gas pressure 40 psi, ESI source voltage 3500 V, fragmentor voltage 140 V, collision gas $N_2$.

### 2.4 Chromatographic conditions for RP column

The separation was performed using a C8 column LiChroCART® 125-4 LiChrospher® 100 RP-8 (125 x 4,0 mm, 5 µm) along with a guard column (LiChrospher® 100 RP-8, 5 µm, 4x4 mm), both Merck KGaA (Darmstadt, Germany). The temperature of the column was maintained at 35 °C. The mobile phase A consisted of a 10 µmol/mL ammonium formate buffer with pH 3.1 (adjusted with formic acid) and mobile phase B consisted of 100 % acetonitrile. The flow rate of the mobile phase was set at 0.4 mL/min. The following gradient elution was used: initial composition was set at 15 % of B and kept isocratic for 10 minutes, after that the composition was raised to 100 % of B during 2 minutes, kept at a 100 % level of B for 4 minutes, and then lowered to the initial 15 % of B during 2 minutes. The column was reconditioned with the initial composition of the mobile phases for 5 minutes with the flow rate of 0.8 mL/min. The injection volume was 10 µL.

### 2.5 Chromatographic conditions for HILIC column

The separation was performed using a HILIC column SeQuant® ZIC®-HILIC 3.5 µm, 2.1 x 100 mm (Merck KGaA) with a guard column Nucleodur HILIC 5µm, 4x2 mm (Macherey-Nagel, Duren, Germany). The same temperature, mobile phase constituents, and flow rate were used as in case of the RP-column. The following gradient elution was used: initial composition was set at 97 % of B and kept isocratic for 2 minutes, after that the composition was changed to 10 % of B during 4 minutes, kept at a 10 % level of B for 4 minutes, and afterwards changed to the initial 97 % of B during 1 minute. The column was reconditioned with the initial composition of the mobile phases for 5 minutes with the flow rate of 0.4 mL/min. The injection volume was 1 µL.

## 3. Results and discussion

Three HPLC methods were systematically optimized (optimum parameters are summarized in sections 2.3-2.5), validated (validation data are summarized in Tables 1 and 2) and compared to each other in order to establish a new highly effective and reliable method for the determination of thiopurine S-methyltransferase activity suitable for the routine clinical use. A conventional approach is represented by the RP-HPLC-UV method, advanced approach by RP-HPLC-MS method, and the new progressive approach by the HILIC-HPLC-MS method.

### 3.1 Method development

While most of the published methods used a $C_{18}$ RP-column [18,19,22–29], we preferred a $C_8$ RP-column in order to improve separation of 6-MMP from the matrix constituents. This is critical when using non-selective UV detection. The retention times of 6-MMP in the published RP-HPLC methods ranged between ca. 4 and 27 min [22,27] while in our case it was 9.3 min. The optimized $C_8$ RP-HPLC-UV method was suitable for the identification of 6-MMP as well as 6-MP, however, some partial overlapping could not be avoided as it can be seen in Fig. 1. The presence of such interference makes the evaluation of the obtained data difficult and can be source of inaccuracies in the results of the assay.

Therefore, in the second step, $C_8$ RP-HPLC was hyphenated with MS in order to enhance detection selectivity. The optimized chromatographic separation conditions were the same as those used in the RP-HPLC-UV method (section 2.4). The use of MS detection significantly enhanced orthogonality of the analytical system as MS hyphenation provided specific detection based on different m/z values of the analyzed compounds. Therefore, interferences of the analytes with compounds originating from the sample matrix, as they were seen in RP-HPLC-UV, were prevented when using the RP-HPLC-MS method, see Fig. 2. On the other hand, the shapes of some peaks were not optimum. For example, a splitting of

6-MP peak was observed, probably due to an interaction with the matrix in the ion source (see the elution position of 6-MP in the TIC chromatogram, upper panel in Fig. 2). It is necessary to mention, however, that poor peak shape of 6-MP (substrate) has no relevance in the determination of TPMT activity based on 6-MMP (enzymatic product) quantification. On the other hand, we suppose information in varying amount of the original substrate (6-MP) in incubated RBC samples could be useful for further investigation of the thiopurine metabolism in different biological systems (e.g. different patients).

In the third step, the chromatographic conditions were modified to be more favorable with respect to the elution parameters of the analytes and ESI-MS compatibility. A method employing the HILIC column coupled with the MS detection was developed as an alternative to the RP-HPLC-MS. To our best knowledge, this is the first method that uses this type of column for the TPMT activity determination. The HILIC column, providing polar interactions with the analytes, was responsible for considerable changes in separation selectivity (e.g. reversing migration order of 6-MMP and 6-MP). Applying higher concentration of less polar organic phase (acetonitrile) in HILIC separation was favorable for the combination with MS, so that significantly higher detection responses of the analytes were obtained in comparison with the RP-HPLC-MS approach. Moreover, the shapes of the analytes peaks were improved as it can be seen in Fig. 3. Very important benefit of the HILIC separation was considerable shortening in the 6-MMP retention. By using this column, the obtained retention time of 6-MMP was 1.28 min that was several times lower value than the lowest value obtained in the TPMT activity studies so far, e.g. 4.42 min in ref. [16]. In comparison to our RP-HPLC-UV/MS methods (described above) the total analysis time, including reconditioning of the column, for the HILIC-HPLC-MS method was lower by 7 minutes.

### 3.2 Evaluation of performance parameters

Performance parameters of the optimized RP-HPLC-UV, RP-HPLC-MS, and HILIC-HPLC-MS methods were evaluated for the 6-MMP standard present in the model (spiked) RBC lysate matrix, and the resulting data are given in Table 1. The precise mass of 6-MMP, found by the high-resolution Q-TOF MS, was 167.0386 (m/z $[M+H]^+$ ± 20 ppm). The performance parameters were evaluated according to the ICH guideline [30].

Linearity, tested in the concentration range up to two decadic orders and expressed via coefficient of determination ($r^2$), was acceptable for the analyte measured in the RBC lysate matrices. This range was suitable also for the concentration levels of 6-MMP monitored during the TPMT activity determination.

The limits of detection (LOD) and quantitation (LOQ) for 6-MMP in the injected samples (prepared according to the standard procedure given in sections 2.2.2 and 2.2.3), calculated from the calibration lines as $3.3\sigma_a/b$ and $10\sigma_a/b$, respectively, were in sub μmol/mL level when using RP-HPLC-UV/MS methods, while they were ca. 20-times lower when using HILIC-HPLC-MS method. This highlighted usefulness of the HILIC-HPLC-MS method in the ultra-trace determination of 6-MMP in RBC lysates (and, by that, to determine lower TPMT activities) and in the analysis of reduced amounts of the sample (lower consumption of biological material). Although LOD/LOQ values presented as nmol/mL demonstrated limits of given analytical method in determination of 6-MMP in 1 mL of injected sample, in clinical practice these limits are usually presented as nmol/mL RBC including correction (i.e. increasing) of these values for given RBC dilution (done during sample preparation procedure). The later expression is practical for clinicians as it refers to minimum TPMT activity (nmol/mL RBC/h) that can be determined under real sample preparation conditions in clinical laboratory. Although LOD/LOQ (nmol/mL RBC) of the HILIC-HPLC-MS method can be additionally improved (injecting 10-times less diluted samples), the given value reflects practical requirements of the present work (i.e. expected higher TPMT activities in healthy volunteers and, by that, need for additional sample dilution to be analytical signal in the linear range of the calibration line).

Ionization suppression due to coeluting matrix (i.e. matrix effect) was evaluated by comparing peak areas obtained after direct injection of the samples into MS and corresponding with 6-MMP spiked into the mobile phase and into the RBC lysate matrix, see the left panel in Fig. 4. The obtained value (103.09 % in favor of the RBC lysate matrix) indicated negligible/low matrix effects when determining 6-MMP in the RBC lysate samples.

From the data in Table 1 it is apparent that the proposed methods provided excellent precision of the analyses, where RSD of migration times and peak areas did not exceed 1.0 and 2.0%, respectively. The results were comparable and consistent for all the methods tested. The findings in this paragraph approved the methods for the practical highly reproducible measurements.

The analysis of the QC samples on the low, medium and high (i.e. 0.1, 0.5, 1 nmol/mL, respectively) concentrations of the spiked 6-MMP verified the accuracy of the three methods in the TPMT activity determination. The average recoveries for expected concentration levels of 6-MMP in clinical samples were 92.46, 98.45, and 97.23% in case of RP-HPLC-UV, RP-HPLC-MS, and HILIC-HPLC-MS methods, respectively. The HPLC-MS methods provided consistent and more accurate results than the HPLC-UV method. Detail data obtained from the recovery test using the HILIC-HPLC-MS method are summarized in Table 2. Generally, the recovery test approved acceptable accuracy of all the examined methods and, by that, their suitability for the practical use in the RBC lysate matrices.

As expected, the HPLC-MS methods have superior specificity when compared to the HPLC-UV method. The influence of the co-eluting peaks originating from the matrix was eliminated due to specific MS response towards to the analyte. Therefore, the HPLC-MS approach is recommended for highly reliable analyses of 6-MMP in the RBC lysate matrices.

Taking into consideration all the performance parameters, the highest criteria were fulfilled by the HILIC-HPLC-MS method. This method was superior (in comparison with RP-HPLC-UV/MS methods) especially in parameters of sensitivity, selectivity, analysis time, and sample consumption. Hence, usefulness of this new method for practical routine use in the TPMT activity determination was clearly confirmed.

### 3.3 Method application

All three of the presented methods have been applied for the determination of TPMT activity in the RBC lysates of two healthy volunteers and the results were compared. It was found out that the TPMT activity of the first and the second volunteer ranged in the intervals of 10.59 – 10.86 nmol/mL RBC/h and 10.62 – 12.18 nmol/mL RBC/h, respectively. These values refer to a nmol/mL concentration of enzymatically produced 6-MMP corrected for the given RBC dilution (as stated in sections 2.2.2 and 2.2.3). The results obtained from the three methods were comparable and consistent that confirmed their good accuracy and usefulness for the routine clinical use.

In addition to the evaluation of matrix effects described in section 3.2, the results obtained from the calibration curve were compared with those obtained by the method of standard addition where any matrix effects are compensated (eliminated). For the records illustrating additions of the 6-MMP standard to a volunteer RBC lysate sample, see the right panel in Fig. 4. The differences (relative errors) between the TPMT activity determined in the volunteer samples by the calibration curve and by the method of standard addition ranged in the interval of -2.18% – 0.46%. These results confirmed negligible/low influence of the matrix effects on the determination of TPMT activity in RBC lysates, and, by that, usefulness of the proposed method for its practical applications.

Anyway, the HILIC-HPLC MS method was suitable not only for the faster and more sensitive determination of 6-MMP, but also for the highly reliable monitoring of the original substrate (6-MP), as it can be seen in Fig. 5. It can be valuable for the simultaneous determination of TPMT activity as well as the level of free 6-MP that serves as a precursor for the in-vivo synthesis of the active thioguanine nucleotides. As the levels of free 6-MP can differ among patients (see also profiles of two volunteers in Fig. 5), it would be interesting to investigate relationship between these levels and real therapeutic profit of thiopurines on different patients. In this way, a more complex view on the thiopurine metabolism can be easily obtained yet before starting the thiopurine therapy. In order to see benefits of such complex analytical information, however, the evaluation of TPMT activity in the large groups of patients prior thiopurine treatment will be needed.

## 4. Conclusion

Three HPLC methods, namely RP-HPLC-UV using traditional concept, RP-HPLC-MS using advanced detection, and HILIC-HPLC-MS using new progressive concept combining advanced separation and detection, were developed for the determination of TPMT activity in RBC lysates.

The performance parameters clearly demonstrated that the HILIC-HPLC-MS method was superior not only over traditional RP-HPLC-UV method but also over advanced RP-HPLC-MS method used for the TPMT activity determination. It is well documented especially via improved sensitivity (determination of lower TPMT activity), selectivity (minimized interferences), shortened analysis time (more analyzed samples in time unit), reduced sample consumption (beneficial to patients), i.e. parameters playing key role in routine bioanalysis. Therefore, the HILIC-HPLC-MS method is proposed as a new progressive method in the routine clinical TPMT activity determination.

In addition, the HILIC-HPLC-MS method enables the simultaneous and highly reliable determination of TPMT activity as well as the level of free 6-MP that serves as a precursor for the in-vivo synthesis of the active thioguanine nucleotides. In this way, a more complex view on the thiopurine metabolism can be easily obtained yet before starting the thiopurine therapy. This potential for the clinical practice will be evaluated in our next work including statistically significant groups of IBD patients.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethical approval:** This work and all its experiments, including the sample collection from humans, were approved by the Medical Ethical Committee of St Michael`s Hospital, Bratislava, Slovakia.

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## Figure captions

**Figure 1:** Representative profiles of the incubated RBC sample and the blank matrix obtained by the RP-HPLC-DAD method. The blank matrix (blue) was prepared in the same manner as the RBC sample (green) but without 6-MP (see sections 2.2.2 and 2.2.3). For the working conditions see sections 2.3 and 2.4. SAM – S-adenosyl-L-methionine; 6-MP – 6-mercaptopurine; 6-MMP – 6-methylmercaptopurine.

**Figure 2:** Representative profiles of the incubated RBC sample and the blank matrix obtained by the RP-HPLC-MS method. Upper panel: TIC chromatograms. The blank matrix (blue) was prepared in the same manner as the RBC sample (green) but without 6-MP (see sections 2.2.2 and 2.2.3). Lower panel: EIC chromatograms extracted according to following m/z [M+H]+ of the analytes (± 20 ppm): 167.0386 (6-MMP), 155.0112 (Cl-P), 153.0226 (6-MP), 399.1450 (SAM). For the working conditions see sections 2.3 and 2.4. 6-MMP – 6-methylmercaptopurine; 6-MP – 6-mercaptopurine; SAM – S-adenosyl-L-methionine; Cl-P – 6-chloropurine.

**Figure 3:** Representative profiles of the incubated RBC sample obtained by the HILIC-HPLC-MS method. EIC chromatograms extracted according to following m/z [M+H]+ of the analytes (± 20 ppm): 167.0386 (6-MMP), 155.0112 (Cl-P), 153.0226 (6-MP), 399.1450 (SAM). For the sample preparation see sections 2.2.2 and 2.2.3. For the working conditions see sections 2.3 and 2.5. 6-MMP – 6-methylmercaptopurine; 6-MP – 6-mercaptopurine; SAM – S-adenosyl-L-methionine; Cl-P – 6-chloropurine.

**Figure 4:** EIC profiles used for the evaluation of matrix effects. Left panel: analytical signals (peaks) of 6-MMP (1 nmol/mL) spiked into the mobile phase and into the RBC matrix. The samples were injected directly into MS (1 μl injection volume). Right panel: analytical signals (peaks) of 6-MMP spiked in different concentrations into the incubated RBC sample obtained from the volunteer 2. The samples were injected into HILIC-HPLC-MS (1 μl injection volume). EIC chromatograms extracted according to following m/z [M+H]+ of 6-MMP (± 20 ppm): 167.0386. For the sample preparation see sections 2.2.2 and 2.2.3. For the working conditions see sections 2.3 and 2.5. 6-MMP – 6-methylmercaptopurine.

**Figure 5:** EIC profiles of the incubated RBC samples of two volunteers illustrating advanced TPMT determination by the HILIC-HPLC-MS method. EIC chromatograms extracted according to following m/z [M+H]+ of the analytes (± 20 ppm): 167.0386 (6-MMP), 153.0226 (6-MP). For the sample preparation see sections 2.2.2 and 2.2.3. For the working conditions see sections 2.3 and 2.5. 6-MMP – 6-methylmercaptopurine; 6-MP – 6-mercaptopurine.

**Table 1:** Performance parameters of three different HPLC methods for determination of 6-MMP in spiked RBC lysate matrix[a]

| Parameter | RP-HPLC-DAD | RP-HPLC-MS | HILIC-HPLC-MS |
|---|---:|---:|---:|
| Linearity range (nmol/mL) | 0.1 – 10.0 | 0.1 – 5.0 | 0.01 – 1.0 |
| $\overline{t_R}$(min.), n = 12 | 9.2605 | 9.3726 | 1.2813 |
| $SD_{tR}$ (min.), n = 12 | 0.0176 | 0.0193 | 0.0049 |
| $RSD_{tR}$ (%), n = 12 | 0.1903 | 0.2057 | 0.3854 |
| $RSD_{peak\ area}$ (%), n = 6 | 0.8332 | 1.1829 | 0.5458 |
| Slope (b) | 108.5 | 3468952.6 | 727274.6 |
| $SD_b$, n = 12 | 0.9458 | 42776.5 | 2560.0 |
| Intercept (a) | -2.2933 | 501412.2 | 260.6 |
| $SD_a$, n = 12 | 4.9114 | 109075.8 | 1203.1 |
| Coefficient of determination ($r^2$) | 0.9991 | 0.9988 | 0.9999 |
| LOD[b] (nmol/mL), $3.3\sigma_a/b$ | 0.1494 | 0.1038 | 0.0055 |
| LOD[c] (nmol/mL RBC) | 1.4937 | 1.0376 | 0.5459 |
| LOQ[b] (nmol/mL), $10\sigma_a/b$ | 0.4526 | 0.3144 | 0.0165 |
| LOQ[c] (nmol/mL RBC) | 4.5263 | 3.1443 | 1.6542 |

[a] For the standard/sample preparation see section 2.2. For the working conditions see sections 2.3 (UV, MS), 2.4 (RP-HPLC) and 2.5 (HILIC-HPLC).

[b] LOD and LOQ calculated for 6-MMP in 1 mL of sample

[c] LOD and LOQ calculated for 6-MMP in 1 mL of RBC (corrected for 10- or 100-time dilution of RBC lysate matrix when using RP-HPLC-DAD/MS or HILIC-HPLC-MS, respectively)

**Table 2:** Accuracy of HILIC-HPLC-MS method for determination of 6-MMP in QC samples[a]

| | Concentration level | | |
|---|---|---|---|
| Parameter | Low | Medium | High |
| Range (min % – max %) | 93.25 – 96.98 | 97.48 – 100.37 | 96.39 – 99.31 |
| Mean (%) | 94.75 | 98.89 | 98.05 |
| RSD (%) | 1.3237 | 1.1115 | 1.0638 |

[a] For the standard/sample preparation see section 2.2. For the working conditions see sections 2.3 (MS), and 2.5 (HILIC-HPLC). The RBC lysate matrix was spiked with 6-MMP in three concentration levels (low, medium, high, 0.1, 0.5, 1 nmol/mL, respectively), each QC sample was measured five times.

Fig 1

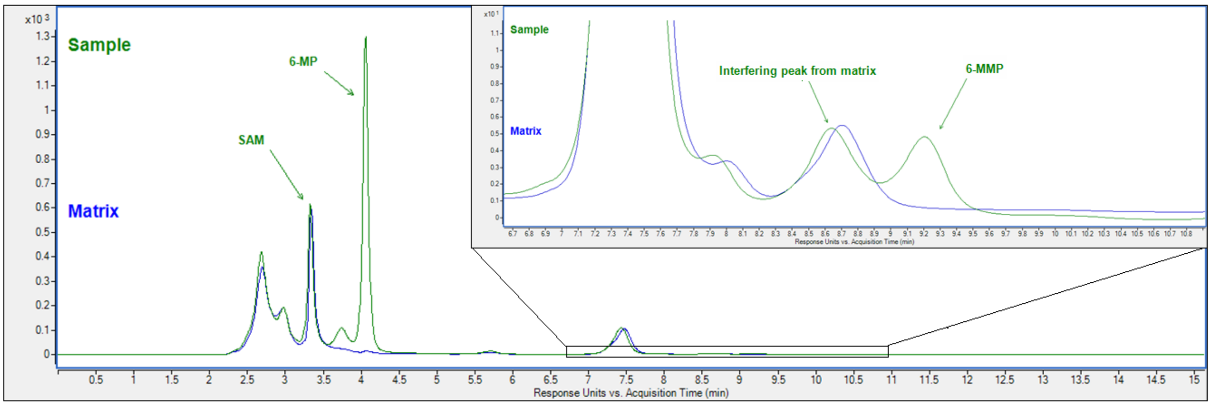

x10 3
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sample
Matrix
SAM
6-MP
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
11
11.5
12
12.5
13
13.5
14
14.5
15
Response Units vs. Acquisition Time (min)
x10 1
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sample
Matrix
Interfering peak from matrix
6-MMP
6.7
6.8
6.9
7
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8
8.1
8.2
8.3
8.4
8.5
8.6
8.7
8.8
8.9
9
9.1
9.2
9.3
9.4
9.5
9.6
9.7
9.8
9.9
10
10.1
10.2
10.3
10.4
10.5
10.6
10.7
10.8
Response Units vs. Acquisition Time (min)

Fig 2

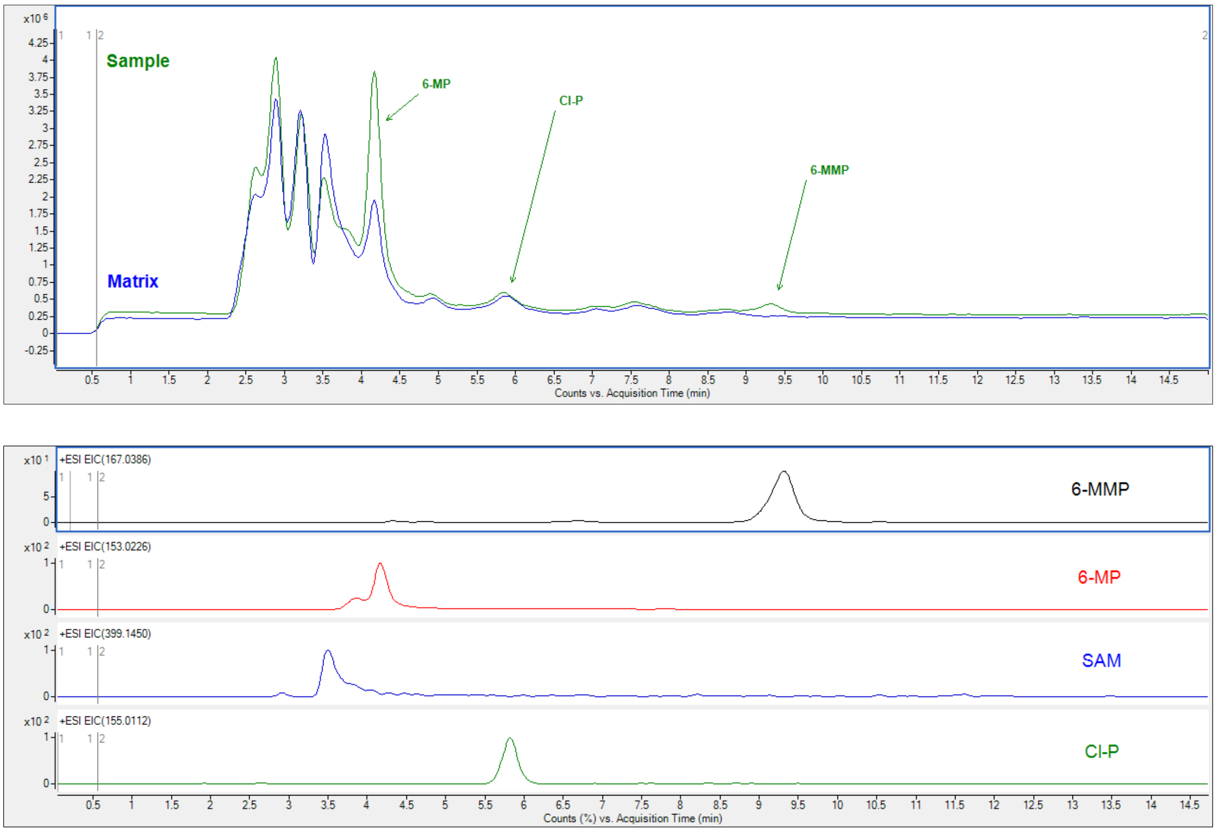

x10 6
Sample
Matrix
6-MP
Cl-P
6-MMP
Counts vs. Acquisition Time (min)
x10 1
+ESI EIC(167.0386)
6-MMP
x10 2
+ESI EIC(153.0226)
6-MP
x10 2
+ESI EIC(399.1450)
SAM
x10 2
+ESI EIC(155.0112)
Cl-P
Counts (%) vs. Acquisition Time (min)

Fig 3

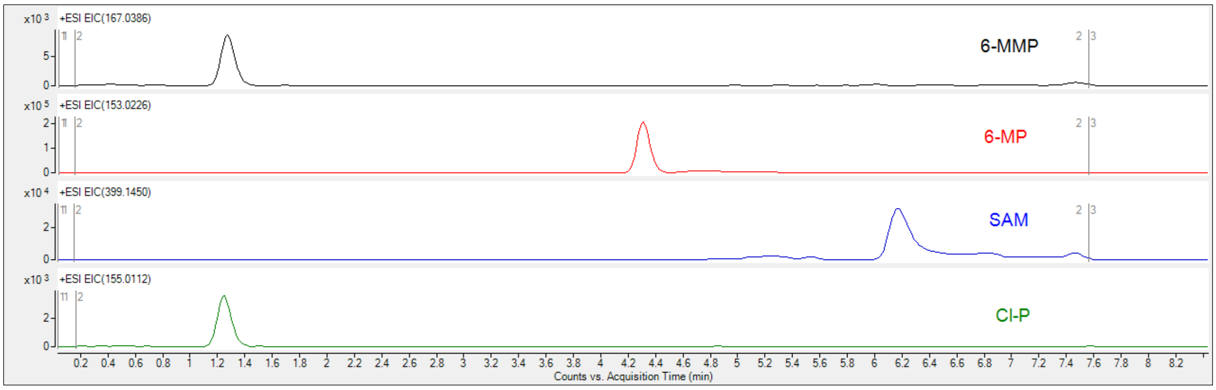

x10 3
+ESI EIC(167.0386)
6-MMP
x10 5
+ESI EIC(153.0226)
6-MP
x10 4
+ESI EIC(399.1450)
SAM
x10 3
+ESI EIC(155.0112)
Cl-P
Counts vs. Acquisition Time (min)

Fig 4

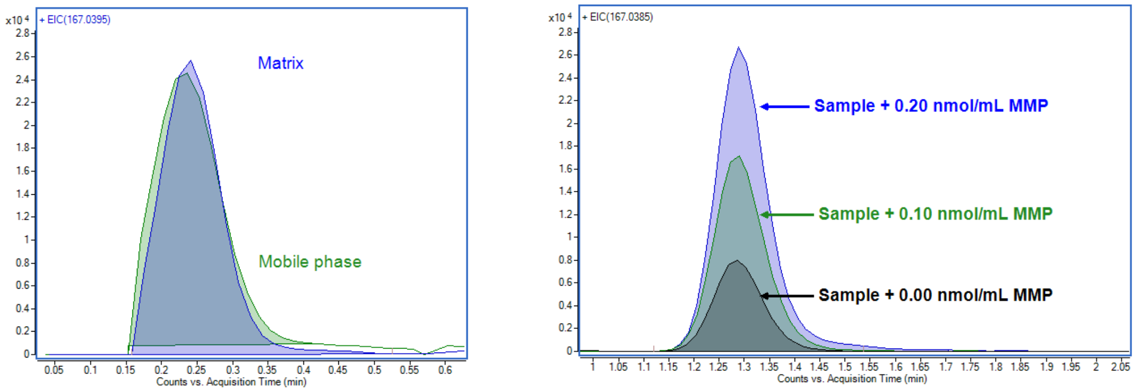
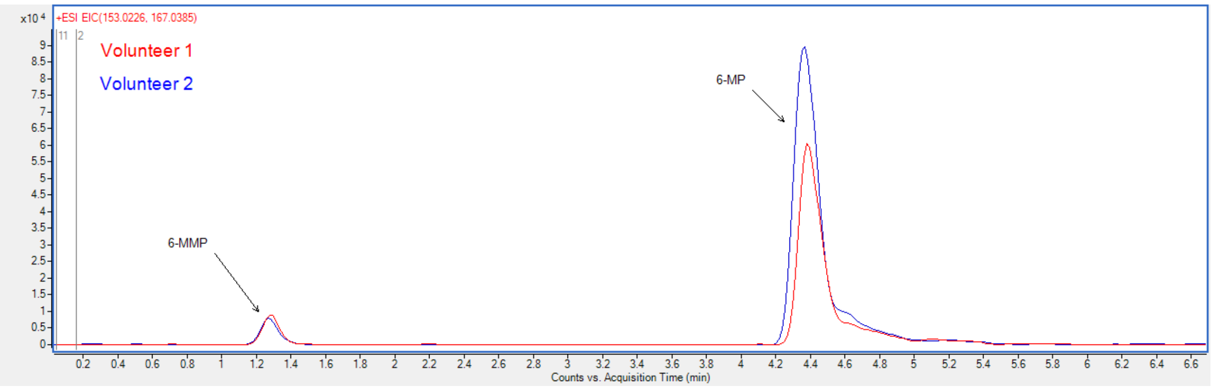

x10 4
+ EIC(167.0395)
Matrix
Mobile phase
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5 0.55 0.6
Counts vs. Acquisition Time (min)
x10 4
+ EIC(167.0385)
Sample + 0.20 nmol/mL MMP
Sample + 0.10 nmol/mL MMP
Sample + 0.00 nmol/mL MMP
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 1.05 1.1 1.15 1.2 1.25 1.3 1.35 1.4 1.45 1.5 1.55 1.6 1.65 1.7 1.75 1.8 1.85 1.9 1.95 2 2.05
Counts vs. Acquisition Time (min)

Fig 5


x10 4
+ESI EIC(153.0226, 167.0385)
11
2
Volunteer 1
Volunteer 2
6-MP
6-MMP
9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
3
3.2
3.4
3.6
3.8
4
4.2
4.4
4.6
4.8
5
5.2
5.4
5.6
5.8
6
6.2
6.4
6.6
Counts vs. Acquisition Time (min)