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A design for external quality assessment for the analysis of thiopurine drugs: pitfalls and opportunities

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Abstract

Background: For the analysis of 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPR), no external quality assessment scheme (EQAS) is currently available and no quality control samples can be made because of the absence of pure substances. An experimental design is tested to compare laboratory analytical results.

Methods: In this EQAS, participating laboratories were asked to select patient samples from their routine analysis and exchange these with a coupled laboratory. Because of large differences in results between laboratories, all standard operating procedures were reviewed, revealing that the origin of these differences could be in the method of hydrolysis and the preparation of calibrators. To investigate the contribution of the calibrators to these differences, one participating laboratory was asked to prepare a batch of calibrators to be shipped to the participating laboratories for analysis.

Results: Results for 6-TGN differed more between laboratories, compared with results for 6-MMPR. For 6-TGN and 6-MMPR 43% and 24% of the results, respectively, were out of the 80%–120% range. When correcting the results

from the exchange of the patient samples with the results of the calibrators, the mean absolute difference for 6-TGN improved from 24.8% to 16.3% ($p < 0.001$), while the results for 6-MMPR worsened from 17.3% to 20.0% ($p = 0.020$).

Conclusions: This first EQAS for thiopurine drugs shows that there is a difference between laboratories in the analysis of 6-TGN, and to a lesser extent in the analysis of 6-MMPR. This difference for 6-TGN can partially be explained by the use of in-house-prepared calibrators that differ among the participants.

Keywords: external quality assessment scheme; harmonization; thiopurine drugs.

Introduction

Thiopurine drugs, azathioprine, 6-mercaptopurine and 6-thioguanine (6-TG), are frequently used in the treatment of inflammatory bowel disease, such as Crohn's disease and ulcerative colitis. Because of great interindividual pharmacokinetic differences, therapeutic drug monitoring is applied to measure 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPR) [1]. The 6-TGN metabolite group consists of 6-thioguanine monophosphate, -diphosphate and -triphosphate

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and are related to the clinical efficacy and toxicity of the thiopurine drugs. The 6-MMPR metabolites are 6-methylthioinosine monophosphate, -diphosphate and -triphosphate and are related to liver and bone marrow toxicity [2]. Since 6-TGN and 6-MMPR metabolites are stored in the red blood cell (RBC) their concentrations are expressed as $\text{pmol}/8 \times 10^8 \text{ RBC}$.

According to the international standard ISO/IEC 15189:2012 [3] with requirements for quality and competence for medical laboratories, each laboratory should participate in interlaboratory comparisons or proficiency testing for all analytes they routinely measure. Since no external quality assessment scheme (EQAS) for thiopurine drugs was available in The Netherlands, the Section Therapeutic Drug Monitoring and Clinical Toxicology (KKG) of the Dutch Foundation for Quality Assessment in Medical Laboratories has started a pilot EQAS for thiopurine drugs. This pilot EQAS is part of the philosophy of Calibration 2.000 [4].

Because ISO/IEC 17043:2010 [5] states that material used in EQAS “should match in terms of matrix, measurand and concentrations, as closely as practicable, the type of items or materials encountered in routine testing or calibration”, no centrally fabricated sample could be prepared due to the unavailability of pure substances of the 6-TGN and 6-MMPR metabolites. In addition, patients using purine drugs could be asked to do a blood donation for production of EQAS samples, but the availability of a sufficient number of donors may be problematic. Therefore, in this pilot EQAS, an experimental design was tested in which patient samples were exchanged between pairs of laboratories instead of a centrally fabricated sample which was sent to all participants.

Materials and methods

Exchange of patient samples

In each EQAS round, each participating laboratory was coupled to one of the other laboratories, creating different laboratory couples in different rounds (e.g. in the first round laboratory 1 is coupled to laboratory 2, in the second round laboratory 1 is coupled to laboratory 3). Each laboratory was asked to select three patient samples from their routine thiopurine samples and to exchange these samples with the coupled laboratory. The laboratory which received the patient sample is defined as the first laboratory, the laboratory that received the samples from the first laboratory for the exchange is defined as the second laboratory. In the seven rounds a total of 11 laboratories participated.

The 6-TGN and 6-MMPR metabolites are unstable in fresh patient samples stored in the refrigerator, but more stable when RBCs are isolated before storage in the freezer [6]. Therefore, the laboratories which obtained the patient samples were instructed to perform the RBC

isolation and count, divide the sample in two aliquots, subsequently freeze the samples and to send one of the frozen samples to the coupled laboratory. With the shipment of the samples, the first laboratory reported the measured RBC counts of the samples to the second laboratory, so that the second laboratory could express the measured 6-TGN and 6-MMPR concentrations in the regular unit $\text{pmol}/8 \times 10^8 \text{ RBC}$. Both laboratories performed the deproteinization and hydrolysis steps on the frozen sample and analyzed the 6-TGN and 6-MMPR metabolite concentrations according to their validated methods.

The exchange of these patient samples was coordinated by the EQAS provider KKG. Before each EQAS round, laboratory couples were formed, and each laboratory was assigned three sample numbers for numbering of the patient samples. Laboratories were provided with instructions and the materials needed for the patient sample exchange, such as tubes, labels with the assigned sample numbers, absorption material, blisters and a label with the address of the coupled laboratory. Upon participation, each laboratory received an insulating Neopor box and a -30°C temperature shell (inside which the patient samples were placed) for distribution of the samples.

Each laboratory reported the 6-TGN and 6-MMPR results of the patient samples to the EQAS provider, together with information about whether the samples were received frozen and the dates of receipt of the samples in the first and second laboratory, RBC isolation and count, 6-TGN and 6-MMPR analysis and shipment. The EQAS provider made a report of the aggregated results.

Participants and measurement methods

All participants reported to use the Dervieux method [7].

Review methods of analysis

Due to large differences in the analytical results between laboratories in the first two rounds, all standard operating procedures were retrieved from the participants for a systematic review. The analytical method used may have great impact on the analytical results. For instance Shipkova et al. [8] reported 1.4–2.6-fold higher 6-TGN results for patient samples analyzed with the Dervieux method [7] compared to the Lennard method [9]. This difference could be the result of (a) differences in the duration of hydrolysis, (b) the concentration and type of acid used for hydrolysis and/or (c) the dithiothreitol (DTT) concentration, which is used for the protection of oxidation of the thiol groups.

Therefore, the first focus in our review of the standard operation procedures was the process of hydrolysis. The second focus of our review was the preparation of the calibrators, because a constant bias was observed between the results of some laboratories possibly attributable to a difference in calibration, and because calibrators are not commercially available but instead are prepared in each laboratory.

Calibrators

Because of differences observed in the standard operating procedures describing the preparation of calibrators, calibrators for 6-TGN

Table 1: Calibrator 6-TG and 6-MMP concentrations.

	Level 1, $\mu\text{mol/L}$	Level 2, $\mu\text{mol/L}$	Level 3, $\mu\text{mol/L}$
6-TG	2.15	3.22	5.01
6-MMP	22.2	29.6	51.7

and 6-MMPR analysis, which were prepared according to the standard operating procedure of one of the participating laboratories, were sent to all participants in the third round of 2015. The participating laboratories were asked to analyze these calibrators in a patient sample run and to calculate the concentration of the received calibrators on their own calibrators.

Since no pure substances for 6-TGN and 6-MMPR metabolites were available, and the metabolites are hydrolyzed to 6-TG and 6-methylmercaptopurine (6-MMP) in the analytical process, the pure substances 6-TG and 6-MMP were used for the preparation of the calibrators.

The calibrators were produced as follows: blood was drawn from a healthy volunteer, not using any of the thiopurine drugs, in lithium-heparin tubes. The samples were washed according to the standard operating procedure for patient samples containing 6-TGN and 6-MMPR, an RBC count was performed and if necessary the RBC concentration was adjusted to $4.0\text{--}4.5 \times 10^{12}$ RBC/L with phosphate buffered saline. The matrix was then spiked with 6-TG purchased from Sigma (Saint Louis, MO, USA) and 6-MMP purchased from TRC (Toronto, Ontario, Canada). A 6-TG stock solution was prepared by dissolving 11.96 mg of 6-TG in 4.0 mL 0.1 mol/L sodium hydrochloride and diluted with water for injections to 50.0 mL. A volume of 2.0 mL of this stock solution was diluted to 20.0 mL with distilled water, creating a 6-TG stock solution of 143 $\mu\text{mol/L}$. 10.00 mg of 6-MMP was dissolved in 4.0 mL 0.1 mol/L sodium hydrochloride, after which 10.0 mL 0.1 mol/L hydrochloric acid was added. This solution was diluted with distilled water to 50.0 mL, creating a 6-MMP stock solution of 1478 $\mu\text{mol/L}$. All chemicals were commercially purchased and of reagent grade.

Three aliquots of washed blank lithium-heparin blood samples of 20 mL were spiked with 0.30, 0.45 and 0.70 mL 6-TG stock solution and 0.30, 0.40 and 0.70 mL 6-MMP stock solution, creating three levels of 6-TG and 6-MMP concentrations (Table 1). Samples were frozen and shipped in temperature shells to the participants.

Data analysis

With this design for external quality control, no reference value can be assigned to the exchanged patient samples, therefore the reported results cannot be related to a true value. The reported results can only be related to each other, and no judgment about good or bad performance can be made from the results in this EQAS. Results of the patient sample exchange were expressed as the result of the second laboratory as the percentage of the result of the first laboratory.

A 20% deviation (80%–120%) was chosen to be acceptable, based the EMA guideline on bioanalytical method validation [10] and previous set limits around true values in EQAS [11–13]. The EMA guideline sets a limit of 15% for accuracy for the entire concentration range but a 20% limit for the lower limit of quantification (LLOQ). In this EQAS, 20% deviation was chosen because preferably one deviation limit is applied, and the most mild one was selected.

Results

In 2014, three rounds of patient sample exchanges were organized. Seven laboratories participated in the first round; therefore two laboratory couples were formed and the remaining three laboratories exchanged samples in a triangular approach. In both the second and third rounds of 2014, eight laboratories participated. In 2015, four rounds were organized with 10 participating laboratories in the first three rounds, and 11 participants in the fourth round. In the first seven rounds, a total of 192 patient samples were exchanged. Out of 192 patient samples, 147 were received in a frozen state by the second laboratory and included in the analysis.

Patient sample exchange

The 6-TGN and 6-MMP results of the first seven rounds of patient sample exchange are depicted in Figures 1 and 2. A line of identity is displayed to obtain a clear picture of the correlation between the result of the first laboratory which obtained the patient sample, and the corresponding result of the second laboratory. Two dotted lines are displayed to indicate the 20% deviation ranges from the line of identity.

Large differences existed between laboratories for the 6-TGN results. For 6-TGN and 6-MMP of four and 23 samples, respectively, at least one of the values was reported as smaller than LLOQ or larger than upper limit of quantification, and therefore, no percentage could be calculated. These results were discarded from

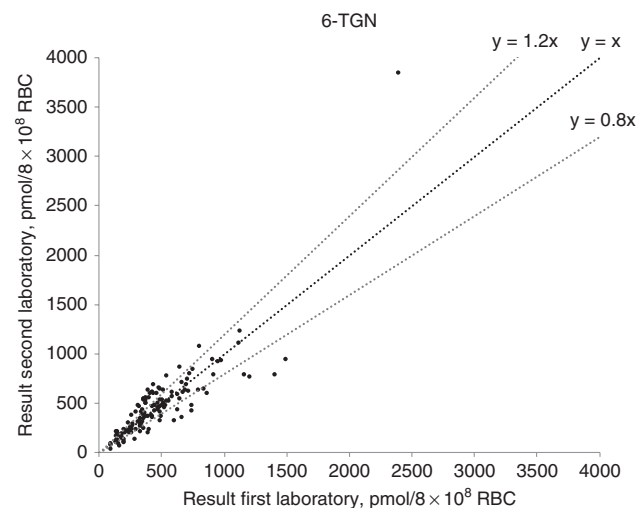


Figure 1: 6-TGN results from seven rounds of patient sample exchange between laboratories.

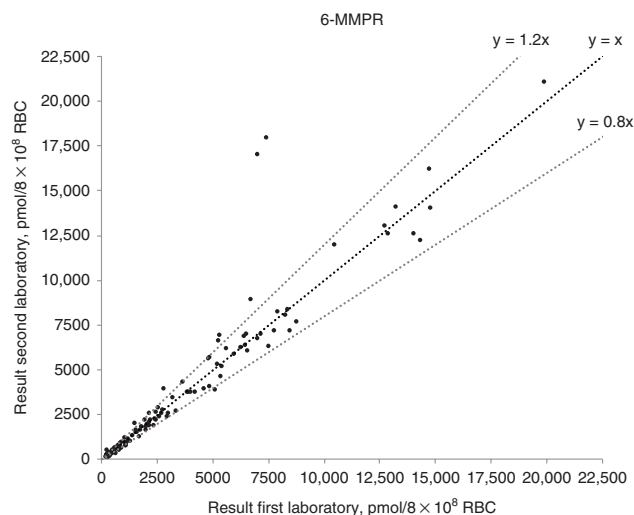


Figure 2: 6-MMPR results from seven rounds of patient sample exchange between laboratories.

the analysis. For one sample, the results for 6-TGN and 6-MMP reported by the second laboratory were, respectively, 266 and 294 times higher, and both were identified as a visual outlier.

For 6-TGN, 61/142 (43%) paired results were outside the 80%–120% ranges. In contrast, 6-MMPR results were more comparable: 30 (24%) of 123 paired results were outside the 80%–120% ranges.

Review method of analysis

The review of the analytical methods used revealed several differences between laboratories. Main differences observed (among other small differences) were (1) the amount of acid used in the denaturation of the proteins in the patient RBC, (2) the concentration and volume of the DTT solution added to patient sera, and (3) the preparation of the calibrators. The main differences between laboratories are described in Table 2.

Calibrators

The centrally prepared calibrators were sent to 10 laboratories. One laboratory did not analyze the samples and one laboratory received the samples after 3 days, resulting in

Table 2: Main differences in amount of acid, DTT concentration and calibrator matrix and preparation in the analysis of 6-TGN and 6-MMPR.

Laboratory	Acid	DTT conc., mM	Calibrator matrix	Calibrator preparation
1	11% Perchloric acid 70%	72	Left over lithium-heparin blood samples used for other analysis (without 6-TGN and 6-MMPR)	Centrifuge, discard plasma and buffy coat, store in freezer and spike with 6-TG and 6-MMP before analysis
2	8% Perchloric acid 70%	50	Fresh, blanc lithium-heparin blood samples from healthy volunteers	Wash according to the SOP for patient samples, store in freezer and spike with 6-TG and 6-MMP before analysis
3	12% Perchloric acid 70%	60	Erythrocytes in SAGM (saline, adenine, glucose, mannitol), purchased from the national blood bank	Dilute in a 1:1 ratio with PBS, spike with 6-TG and 6-MMP and store in freezer
4	14% Perchloric acid 70%	60	Fresh, blanc lithium-heparin blood samples from healthy volunteers	Wash according to the SOP for patient samples, spike with 6-TG and 6-MMP and store in freezer
5	13% Perchloric acid 70%	66	Erythrocytes in SAGM, purchased from the national blood bank	Dilute in a 1:1 ratio with PBS, spike with 6-TG and 6-MMP and store in freezer
6	12% Perchloric acid 70%	60	Fresh, blanc lithium-heparin blood samples from healthy volunteers	Wash according to the SOP for patient samples, store in freezer and spike with 6-TG and 6-MMP before analysis
7	12% Perchloric acid 70%	60	Erythrocytes in SAGM, purchased from the national blood bank	Dilute in a 1:1 ratio with PBS, spike with 6-TG and 6-MMP and store in freezer
8	13% Perchloric acid 70%	61	Water	Spike with 6-TG and 6-MMP before analysis

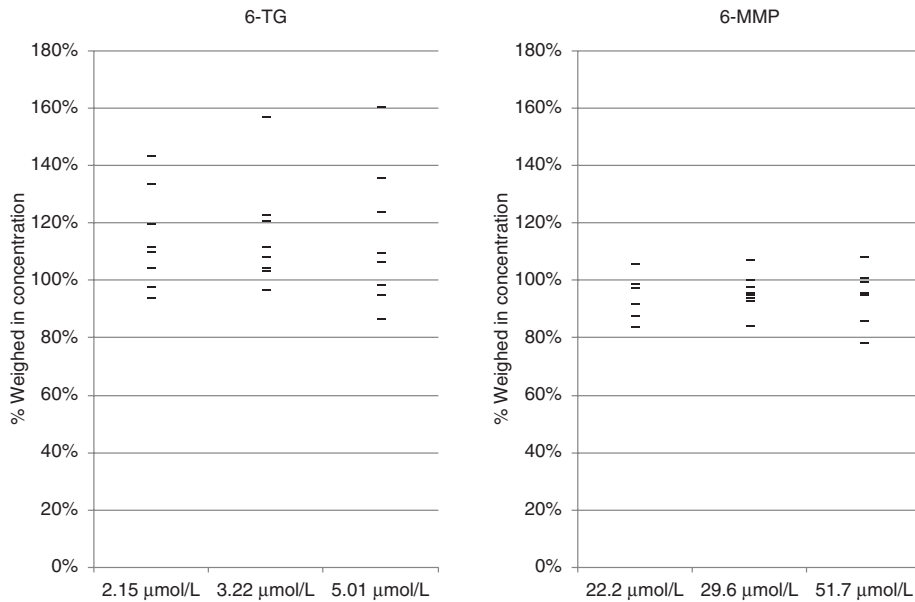


Figure 3: Results for 6-TG and 6-MMP in three calibrator samples.

thawed samples and degraded 6-TG and 6-MMP. Results of the remaining eight laboratories are depicted in Figure 3. The results are comparable to the results which were observed in the exchange of patient sera; results for 6-TG differ greatly among laboratories while 6-MMP results are more comparable between laboratories.

The results from the former analysis of the calibrators indicate that the use of different calibrators could contribute to the observed differences in patient sera results. To test this hypothesis, the results of the calibrators were used to correct the patient sera results to assess the contribution of the use of different calibrators to the differences in results for 6-TGN and 6-MMPR between laboratories. Because two laboratories did not report results for the calibrators and one laboratory received thawed and degraded samples, 47 6-TGN and 43 6-MMPR results could not be corrected for the results of the calibrators.

The difference between the results of the first and second laboratory was expressed as the absolute percentage of the result of the first laboratory. The absolute percentages were compared before and after correction for the calibrator results. The results for 6-TG improved and the results for 6-MMP worsened after correction (Table 3).

Comparison of the mean within-laboratory variances with the overall variances showed significant differences for both 6-TG (0.017 vs. 1.995, $p < 0.001$) and 6-MMP (0.527 vs. 30.14, $p < 0.001$), indicating that the overall variances are mainly determined by the between-laboratory variances.

Discussion

The results of this first EQAS for the analysis of thiopurine drugs show that there is a large interlaboratory variation in the analytical results for 6-TGN. For 6-MMPR, the results between the laboratories differ less. For the analysis of 6-TGN, the use of in-house-prepared calibrators among the laboratories seems to contribute for approximately 34% of this difference. Because the overall variances for both 6-TGN and 6-MMP in the calibrator samples are primarily determined by the between-laboratory variances, our hypothesis was that the differences seen in the results of the patient samples would diminish. This effect was only seen in the 6-TGN results, possibly due to non-commutability of the calibrator for the 6-MMPR analysis or due to differences or non-specificity in the applied methods,

Table 3: Mean absolute differences (%) for 6-TG and 6-MMP in patient samples before and after correction for the calibrator results.

	n	Before correction	After correction	Difference	95% CI	p-Value
6-TG	95	24.8	16.3	8.5	4.4–12.8	<0.001
6-MMP	80	17.3	20.0	–2.7	–5.0 to –0.4	0.020

despite the fact that the participants claim to use the same method and apply the same therapeutic range.

A previous study by Shipkova et al. [8] showed that 6-TGN results were different between two methods of analysis, due to the differences in the duration of hydrolysis, the concentration and type of acid used for hydrolysis and/or the DTT concentration. The participating laboratories in this study all claimed to apply the Dervieux method, but during the review of the used analytical methods, deviations from the publication of Dervieux [7] were seen in the amount of acid and DTT used. These differences in the amount of acid and DTT used could be a (partial) explanation for the residual difference after correction for the calibrator.

Due to the use of patient samples in this EQAS and the lack of a reference method and certified reference material, no statement can be made about the accuracy of the results of the participating laboratories, only a statement about the results of the laboratories compared to each other can be made. This is a weak point of the study, but inevitable since there is no certified reference material available. On the other hand, this study is able to demonstrate the differences between laboratories, and it demonstrates the urgent need for harmonization.

Due to stability reasons and the RBC count, a part of the pre-treatment of the samples could only be performed by the first laboratory before the frozen samples could be sent to the second laboratory. This is not ideal in an EQAS since preferably the entire analytical process is included, but inevitable in this design and the characteristics of the analytes. The use of calibrators as EQAS samples was an improvement compared to the exchange of patient samples because all laboratories received the same sample and a more solid comparison between laboratory methods could be made, but the disadvantage of the lack of pre-treatment remains.

A disadvantage of both approaches is the instability of the 6-TGN and 6-MMPR metabolites and the shipment of the sample. The amount of variability introduced by the shipment is unknown. The extent of this uncertainty is reduced by only including samples which arrived frozen in the second laboratory. The shipment of the sample is on the other hand a true comparison with an actual patient sample because these are often also shipped to a laboratory for analysis.

An advantage of the use of the calibrators is that the differences between laboratories can be primarily assigned to the analytical process. In the future, a further improvement of this EQAS may be pursued by sending pooled patient material or single patient donations to the participating laboratories.

Even though no true comparison between laboratories can be made for the analysis of thiopurine drugs, this exchange and analysis of patient samples and calibrators is a first report of the differences between laboratory results for thiopurine metabolites. These differences may have a negative impact on patient care when patients switch between different health-care providers and dose adjustments are made according to the results of different laboratories.

Conclusions

This first EQAS for the analysis of thiopurine drugs shows that there is a large difference between analytical 6-TGN results coming from different laboratories whereas 6-MMPR results do not seem to differ in a clinically relevant way. This difference for 6-TGN may partially be explained by the use of in-house-prepared calibrators that differ among the different laboratories. It is recommendable to harmonize the calibrators as a first step to reduce between-laboratory variation. More research is needed to determine which other factors contribute to the differences between laboratories in order to further reduce the between-laboratory variation.

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