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Validation and application of a high-performance liquid chromatographic-based assay for determination of the inosine 5′-monophosphate dehydrogenase activity in erythrocytes

Philipe N. Khalil [a], Norbert Erb [b], Maurice N. Khalil [b,*], Gabriele Escherich [b], Gritta E. Janka-Schaub [b]

[a] *Department of Surgery, Downtown Medical Centre, Ludwig-Maximilians-University, 80336 Munich, Germany*
[b] *Clinic of Paediatric Haematology and Oncology, University Medical Centre, Hamburg-Eppendorf, D-20251 Hamburg, Germany*

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Abstract

Thiopurine drug monitoring has become an important issue in treating children with acute lymphoblastic leukaemia (ALL). In this population, a genetic polymorphism causes wide differences in the activity of thiopurine *S*-methyletransferase (TPMT) – the rate-limiting enzyme of the thiopurine degradation metabolism – leading to the necessity of drug dose adjustments. It is not yet known if similar differences exist in the inosine 5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205), the rate-limiting enzyme of the thiopurine synthesis. To test this, we established and validated a high-performance liquid chromatographic (HPLC)-based assay to determine the IMPDH enzyme activity in erythrocytes. The remarkable features of this assay are its simple erythrocyte separation/haemolysis and assay conditions and a distinct segregation of xanthosine 5′-monophosphate (XMP) from the clear supernatant after precipitation. The probes were processed without a time-consuming extraction and heating procedure and the assay demonstrated a good intra- and inter-day stability as well as a recovery rate of approximately 100%. The IMPDH enzyme activity was measured in erythrocytes of 75 children with diagnosis of ALL before starting antileukaemic therapy and their activity compared to those of 35 healthy adult controls. The measured enzyme activity was wide ranging in both groups. The individual enzyme activity differences observed in children with ALL might lead to different concentrations of the thioguanine nucleotide levels in those undergoing the standard thiopurine dose regimen.

Keywords: HPLC; Enzyme; Inosine-5′-monophosphate dehydrogenase; Thiopurine; Leukaemia

1. Introduction

The thiopurines azathioprine (5-AZA) and mercaptopurine (6-MP) are frequently used during immunosuppressive therapy for leukaemia, rheumatoid arthritis, or inflammatory bowel disease and/or rejection prophylaxis after organ transplantation [1–3]. After oral intake, these pro-drugs have to first be converted into their active metabolites [1,4]. Thiopurine metabolism is characterized by three competitive metabolic pathways, two catabolic routes by the xanthine oxidase and the thiopurine *S*-methyletransferase and one anabolic route, which results in three different 6-thioguanine nucleotides (TGN) via the key enzyme inosine 5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) [1,4,5]. The cytotoxic and immunosuppressive properties of these thiopurines are mediated by inhibition of the synthesis of proteins, DNA and RNA, owing to their reactive *thiol* group [6,7].

The thiopurine 6-MP is the standard drug used in maintenance therapy for children with acute lymphoblastic leukaemia (ALL) [8–10]. In recent years, different authors have focused on thiopurine degradation metabolism by measuring the TPMT enzyme activity. This interest can be attributed to the known genetic allelic polymorphism in the TPMT gene, which causes wide differences in individual enzyme activity to ensure optimal thiopurine therapy [4,11]. This explains why (in children with ALL) the standard thiopurine regimen results in a severe bone marrow depression with lethal outcome if TPMT enzyme activity is low or absent, whereas patients with high enzyme

* Corresponding author. Tel.: +49 40 42803 4829; fax: +49 40 42803 8101.
E-mail address: khalil@uke.uni-hamburg.de (M.N. Khalil).

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activities may be treated at a too low dose [12–16]. Whether or not similar individual differences also exists in IMPDH enzyme activity in children with ALL is not entirely resolved. The reason for this could be that few authors have focussed on the conversion of thiopurines into their active metabolites mediated by the rate-limiting enzyme IMPDH in ALL patients. To our knowledge, only two previous reports have addressed this issue. Price and colleagues applied a radioactive-based chromatographic separation using DE81 cellulose paper and liquid scintillation to bone marrow and peripheral blood lymphocytes in a small series of patients. They observed both a wide range and a higher mean of IMPDH activities in children with leukaemia compared to healthy controls [17]. By contrast, Montero used a non-radioactive high-performance liquid chromatographic (HPLC)-based assay to determine the IMPDH enzyme activity in erythrocytes of patients with leukaemia and lymphomas and detected no enzyme activity differences compared to healthy controls [18].

In our efforts to monitor the thiopurine therapy in children with ALL to ensure an optimal dosage regimen and to reduce adverse side effects, we previously focused on patient's compliance, the thiopurine pharmacokinetics and the degradation metabolism of thiopurines [9,10,19–21]. We consistently focused on the conversion of thiopurine into its active metabolites by measuring its rate-limiting enzyme IMPDH to investigate whether individual differences occur in enzyme activity, similar to that of TPMT. We therefore, established a highly sensitive HPLC-based assay (using separated erythrocytes from banked donor blood) in order to introduce a simple but also sensitive method to prove the interference of various assay steps on the resulting enzyme activity. Basically, the present assay eliminates the need for the lavish separation of the samples by multiple freeze-thawing cycles, the addition of dithiothreitol (DTE) or the need for a dialysis and filtration process. Additionally, no diethylether extraction or heating procedure after incubation of the prepared probes was necessary compared to literature [18].

In our hands, the amount of measurable IMPDH enzyme activity was dependent on the additive and amount of dithiothreitol (DTT) for the necessary redox-protection and KCl for the incubation medium.

After validation the present assay was used to determine the IMPDH enzyme activity in a large group of untreated children with ALL (at the time of diagnosis) and healthy adult volunteers.

2. Materials and methods

2.1. Chemicals

All used reagents were of analytic grade if not otherwise indicated and obtained from Merck (Darmstadt, Germany). Inosine 5′-monophosphate (IMP, F57510, disodium salt × $8H_2O$, Lot 381988/114899), β-nicotinamide adenine dinucleotide (β-NAD^+, N6522, H_2O × acetone, Lot 67H7850), tetrabutylamoniumhydrogensulfate (TBAHS, T-9668, purity of approximately 99%), dithiothreitol (DTT, D-5545) and xanthosine 5′-monophosphate (XMP, X 10030, Lot 63H0019) were obtained from Sigma–Aldrich/Fluka (Taufkirchen, Germany). Purified water was produced with Purelab Plus (USF Seral, Ransbach, Germany). Stock solutions (10.0 mM) of IMP and β-NAD^+ and XMP (2.272 mM) were prepared by dissolving in 0.067 M phosphate buffer pH 7.4 (*Sörensen*: NaH_2PO_4, KH_2PO_4; 81.8:18.2). Stock solutions of DTT (40 mM) and KCl (4 M) were prepared by dissolving in purified water.

These stock solutions were aliquot in 2.0 ml save lock tubes (Eppendorf, Hamburg, Germany) and kept frozen (−21 °C) until usage. The stock solutions were tested routinely during the present investigation and found to be stable (100%) at −21 °C for several months.

2.2. Sample source and preparation of the cell lysate

The assay was established using erythrocytes from banked donor blood. Thereafter, we analysed 100 venous blood samples, 75 untreated children at diagnosis of ALL (53% male, 47% female) and 35 healthy adult volunteers (46% male, 54% female). Patients were recruited by the *COALL* study group, a German multicentre ongoing study for the treatment of ALL in childhood. The age ranged from 1 to 17 years ($\bar{x} = 7$ years) for the patients with ALL and for the healthy volunteers from 18 to 60 years ($\bar{x} = 4$ years), respectively. For morphological and immunological diagnosis and subtyping blood and bone marrow was sent to the central study laboratory located at the Clinic of Paediatric Haematology and Oncology, University Medical Centre Hamburg. All enrolled patients were untreated and none of them received banked donor blood, previously. The investigation was performed in direct accordance with the local ethics committee. A written informed consent was obtained for every patient by their parents.

All samples were collected in EDTA tubes (Sarstedt, Nürnbrecht, Germany) and processed within 24 h. The red cell lysates were processed standardized to measure the IMPDH enzyme activity. The erythrocytes were separated from EDTA blood by *Ficoll* separation or centrifugation at 1000 × *g* (Biofuge pico, Hereus Instruments, Osterode, Germany) at room temperature for 4 min. Plasma and buffy coat were discarded. The red blood cells (RBC) were washed and centrifuged (1000 × *g*) twice with isotonic saline (same volume as discarded). The washed and packed RBC were diluted with water (1:2.5), frozen for complete haemolysis and stored at −21 °C in 2.0 ml tubes (Eppendorf, Hamburg, Germany) until further processing. After complete thawing the RBC lysates were centrifuged for 4.0 min at 16,000 × *g* to remove cell debris. The supernatants were used to measure the IMPDH enzyme activity and haemoglobin content (Hb) (Celldyn 1600, Abbott, Wiesbaden, Germany).

2.3. IMPDH enzyme activity assay

The enzyme activity assay bases on the conversion of IMP to XMP catalysed by IMPDH and β-NAD^+ as hydrogen acceptor. Quadruplicate measurements at a final volume of 500 μl were processed after incubation for 2 h at 37 °C using a dry

heating block (Type 50006101, Liebisch, Bielefeld, Germany). 12.5 μl KCl- and 12.5 μl DTT-stock solution were diluted with 275 μl 0.067 M phosphate buffer (pH 7.4) (resulting in a final concentration of 100 mM KCl and 1 mM DTT). Thereafter, 25 μl IMP- and 25 μl β-NAD^+-stock solution were added (resulting in final concentration of 0.5 mM IMP and 0.5 mM β-NAD^+). The enzymatic reaction was started by adding 150 μl of the prepared RBC lysate. Following incubation the reaction was terminated and protein precipitation induced by 25 μl cold 60% $HClO_4$ for 3.0 min followed by centrifugation for 4.0 min at 16,000 × *g*. A salt precipitation (perchlorate) was inducted by 55 μl K_2HPO_4 (5 M) for 5.0 min and thereby the pH adjusted to 5.4 followed by repeated centrifugation for 4.0 min at 16,000 × *g*. Finally the clear supernatant was analysed by RP-HPLC.

2.4. Chromatography

The RP-HPLC system (Thermo Electron, Engelsbach, Germany) consisted of a pump (P 200), an autosampler (AS 300) with Microvials (Applica, Bremen, Germany, 100 μl) and a variable UV–vis detector (UV 2000) supported by a degasser system (DG 1310 Uniflows Degasys, Tokyo, Japan). The separation was performed at 35 °C on a reversed-phase column using a Hypersil 125 × 3 mm ODS, particle size 5 μm (System Chromcard®, Machery-Nagel, Düren, Germany). The analytical column was protected by a guard column (80 mm × 3 mm, Machery-Nagel, Düren, Germany), which contained the same phase. The flow rate was 0.6 ml/min, the injection volume 10 μl. The mobile phase consisted of 0.025 M sodium phosphate buffer adjusted to pH 5.6 with NaOH (5 M). Thereafter 0.025 M TBAHS was added to the mobile phase. The step-gradient elution [steps: A/B/A] was performed by adding % (v/v) acetonitrile (ACN) to the mobile phase [step: A] (retention time: 0.0–10.0 min), 20% ACN to the mobile phase [step: B] (retention time: 10.1–17.0 min), and again 1% ACN [step: A] (retention time 17.1–26.0). XMP was detected at a wavelength of 254 nm at a retention time of 10.0 min and a total run time of 26.0 min. Finally the chromatograms were traced onto an integrator printer plotter (Chrom Jet tps 100, Thermo Electron, Engelsbach, Germany). The calibration standard (one-point-calibration) used a 1:200 dilution from XMP stock solution prepared as a blank with incubation medium, KCl, $HClO_4$, and K_2HPO_4 but without incubation procedure. The final concentration was 0.01 mM. The calibration graph used 1:100 (0.02 mM), 1:200 (0.01 mM), 1:400 (0.005 mM) and 1:800 (0.0025 mM) dilutions from XMP stock solution as described above.

2.5. Validation of the IMPDH activity assay

The described activity assay was validated by varying the single assay conditions. In particular the optimal incubation process was ascertained by varying the buffer concentration (0.017–0.067 M), the pH adjustment (7.2–7.8), and the used DTT and KCl concentration (0–5 mM and 0–500 mM, respectively). The influence of the $HClO_4$ additive on the concentration of the reaction product was proved by an additional sample using a defined XMP concentration. Different K_2HPO_4 concentrations (0.435–0.551 M) were tested to ensure an optimal perchorate precipitation and pH adjustment for the chromatographic separation. Different commercially available IMP reagents were tested for their product formation concentration (Sigma I 4500 disodium salt, Lot 14H7813; Sigma–Aldriche/Fluka F57510 disodium salt × $8H_2O$, Lot 381988/114899; Sigma S 4625 disodium salt, Lot M3932). Therefore, stock solutions (10.0 mM) of the different IMP Lots were prepared by dissolving in phosphate buffer (pH 7.4) and product formation concentration determined by using the described optimal assay conditions. IMP from Sigma–Aldridch/Fluka (F57510 disodium salt × $8H_2O$, Lot 381988/114899) was the best choice with regard to purity, activity and stability in contrast to that other tested commercial available compounds showed an impurity grade of up to 13%. The linearity and proportionality of the enzymatic reaction regarding the incubation on time was described by different incubation times (0–24 h). Different RBC lysate volumes of one sample (0–200 μl) were taken resulting in different enzyme concentrations in order to describe the linearity and proportionality of the conversion of IMP to XMP. The XMP recovery rate was measured by adding XMP in various concentrations ($n = 6$, 0.0–0.0546 μM) to a sample with a known high haemoglobin concentration (Hb: 12.0 g/dl). Finally, the intra- and interday assay stability was measured, processing frozen aliquots of three erythrocyte samples with known different IMPDH enzyme activities 10 times (intraday assay stability) during 1 day and one of these erythrocyte samples 12 times (interday assay stability) during a period of three months, respectively. Finally different commercially available substrate and co-substrate compounds were analysed for purity, enzymatic activity and solubility under optimal assay conditions.

2.6. Michaelis constants, non-specific XMP formation, kinetic and statistical analysis

Seven different concentrations were used for both, substrate (IMP: 0–1200 μM at a fixed β-NAD^+ concentration of 1200 μM) and co-substrate (β-NAD^+: 0–1200 μM at a fixed IMP concentration of 1200 μM) to determine the saturation curves. For this purpose the usual incubation period of 2 h was extended to 24 h to detect XMP above the baseline noise and eventually non-specific XMP formation in the corresponding blank controls. The saturation curves were the result of the measured enzyme activity minus blank control. The enzyme kinetics analysis of K_M and V_{max} values were estimated by fitting double reciprocal regression (*Lineweaver-Burk Method*) using *Statview*, (Statview, version 5.0.1, SAS Institute Inc., Cary, NC, USA). All other figures used *Software for Scientific Graphing* (Origin, version 6.1, Origin Lab, Northampton, MA, USA). Quadruplicate measurements were done throughout, with exception of the patient/control samples that were analysed in duplicate. All results were expressed as mean ± standard deviation. $\bar{x}$ refers to the mean. For comparison between groups Mann–Whitney *U*-test was used and a $P < 0.05$ considered statistically significant.

3. Results

3.1. Substrate and co-substrate saturation concentration and non-enzyme involved XMP formation

The final saturation concentration of IMP and β-NAD$^+$ was 0.5 mM for both, substrate and co-substrate (Fig. 1a and c). The calculation of K_M and V_{max} resulted in 0.089 mmol/l and 328.8 nmol/gHb/h, for the substrate (IMP) and 0.93 mmol/l and 341.2 nmol/gHb/h, for the co-substrate (β-NAD$^+$), (Fig. 1b and d). There was no non-enzyme involved XMP formation detectable using the described assay conditions in blank controls (Figs. 2 and 3c).

3.2. Enzymatic reaction

The enzymatic reaction was linear up to 24 h and depended on the used RBC lysate volume. The rate of XMP formation remained constant within the incubation period of 24 h and a RBC lysate volume of 0–200 μl (Hb: 8.0 g/dl) and the XMP formation linear and proportional during the incubation period of 0–6 h (t_i: 0, 2, 4 and 6 h) using RBC lysates up to a Hb of 12.3 g/dl (0, 5.5, 7.4, 10.0 and 12.3 g/dl). Blank controls showed no XMP formation (Fig. 2). The optimal assay pH was 7.4 and only minor activity differences were seen between a pH of 7.2–7.8 (C.V. ≤ 3%). In contrast to that a deviation f[rom] the optimal phosphate buffer concentration (0.067 M) resul[ted] in a reduced XMP formation of up to 10%. The optimal DT[T] concentration for redox protection was 1 mM, low[er] or higher concentrations resulted in an increased fall of [the] mea[sur]able

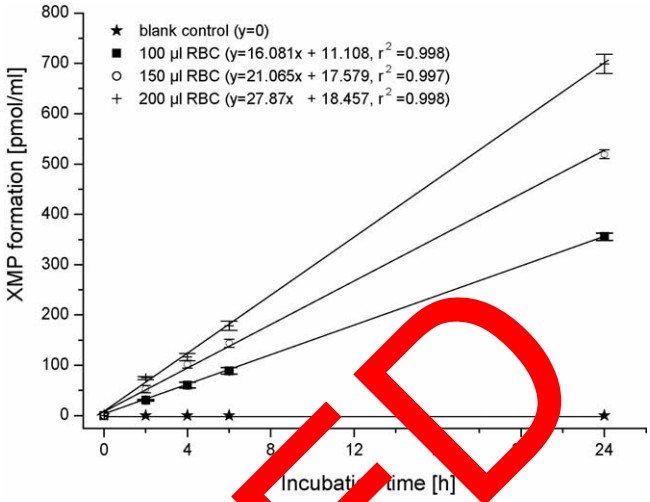

Fig. 2. Linearity and proportionality of the enzymatic reaction from IMP to XMP. Projection of the linearity of the enzymatic reaction in dependence of the incubation time and for the described assay

XMP formation ($n = 8$, $\bar{x}$ = 6.31 − 9.38 nmol XMP/gHb/h, C.V. 33%). A lower XMP formation was detected ($n = 7$, $\bar{x} = 0.79$ − 1.8 nmol XMP/gHb/h, C.V. 37%) if the optimal KCl concentration of 100 mM was not used. The probes were stable for 36 h (n = 0, $\bar{x} = 3.04$ nmol XMP/gHb/h, C.V. 1.6%) after acid precipitation and frozen by −21 °C for at least 3 month ($n = 3$, $\bar{x}$ = 3.22 nmol XMP/gHb/h C.V. 5%). An exact pH adjustment (7.4) and perchlorate precipitation with 55 μl 5 M K_2HPO_4 is warrant to avoid a poor HPLC separation with overlapping peaks.

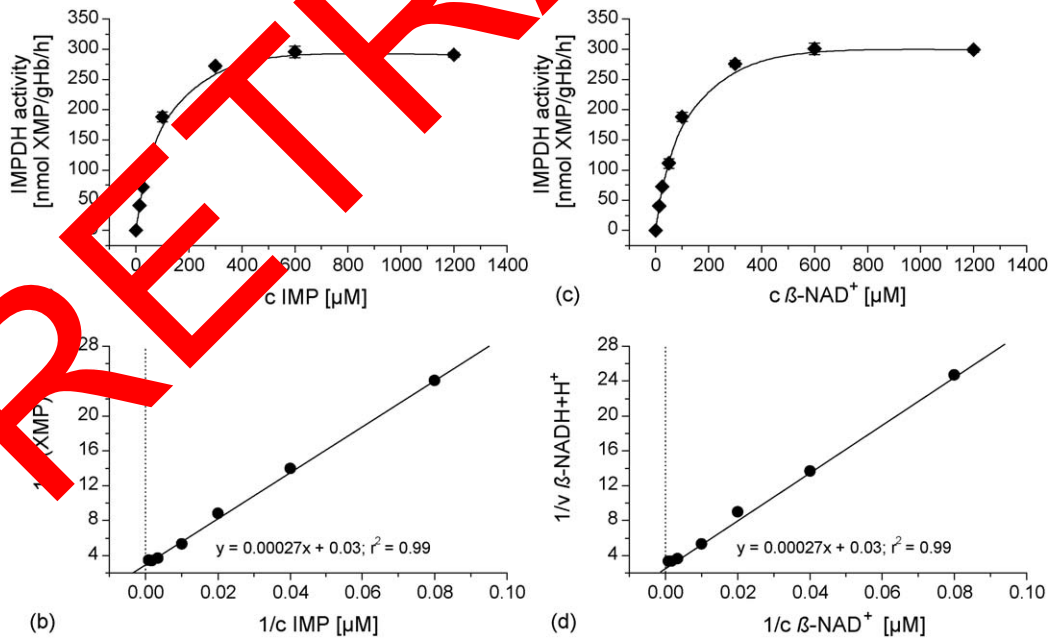

Fig. 1. IMPDH assay kinetics. Substrate (a) and co-substrate (c) saturation curves using (a) 0–1200 μM IMP and a constant co-substrate concentration of 1200 μM β-NAD$^+$ and (c) 0–1200 μM β-NAD$^+$ and a constant substrate concentration of 1200 μM IMP, respectively. The saturation concentration was reached at a substrate concentration of 500 μM IMP (a) and a co-substrate concentration of 500 β-NAD$^+$ μM (c). Double reciprocal plots of the substrate (b) and co-substrate (d) saturation curves for determination of K_M and V_{max} resulting in 0.089 mmol/l and 328.8 nmol/gHb/h, for the substrate (IMP) and 0.093 mmol/l and 341.2 nmol/gHb/h, for the co-substrate (β-NAD$^+$). The usual incubation period of 2 h was extended to 24 h to detect XMP above the baseline noise and eventually non-specific XMP formation in the corresponding blank controls.

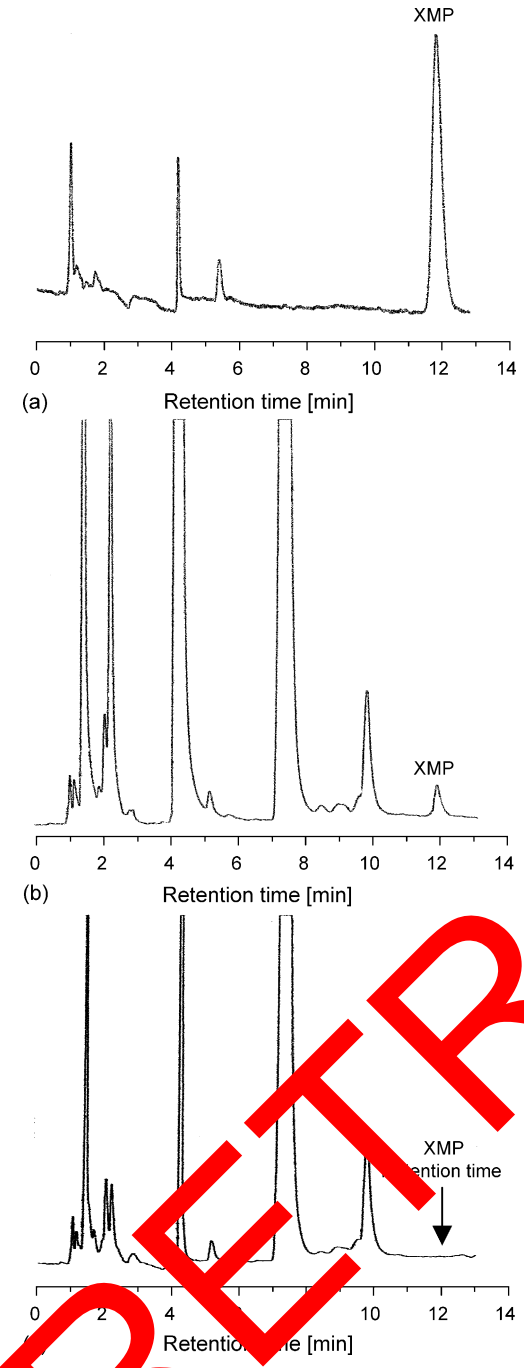

Fig. 3. Chromatographic separation of XMP from erythrocyte lysates. Chromatographic conditions: Hypersil 125 mm × 3 mm, ODS, particle size 5 μm with guard cartridge. The injection volume was 25 μl and the flow rate adjusted to 0.6 ml/min. The mobile phase consisted of 0.025 M sodium phosphate buffer adjusted to pH 5.6 containing 0.025 M TBAHS and ACN. The elution process used a step-gradient [A/B/A] for separation: [A] with 1% ACN (v/v), 0.0–10.0 min; [B] with 20% ACN (v/v), 10.1–17.0 min; [A] with 1% ACN (v/v), 17.1–26.0 min. XMP was measured at a wavelength of 254 nm. Calibration standard (a): XMP stock solution diluted 1:200 with/in incubation-solution containing phosphate buffer pH 7.4 (0.067 M), KCl (100 mM) and DTT (1 mM), and 60% $HClO_4$ (25 μl) and 5 M K_2HPO_4 (55 μl) treatment prepared as describe in material and method. The XMP retention was 12.0 min, the total run time 26 min. Sample (b): Representative sample of an erythrocyte lysate, incubated with IMP and β-NAD^+ as previously described in material and method and reconditioned treatment with $HClO_4$ and K_2HPO_4. Control (c): Control sample of b, prepared and incubated without IMP and β-NAD^+, reconditioned after incubation as described.

Table 1
Assay recovery of XMP using different XMP concentrations up to 0.0546 μM

Used XMP concentration (μM)	Recovery $\bar{x}$ (μM)	SD (%)	Recovery rate (%)
0.0	0.0	0	100
0.0018	0.0018	2.77	100
0.0091	0.0090	3.37	98.9
0.0182	0.0181	2.50	98.6
0.0364	0.0359	0.80	98.9
0.0546	0.0542	1.45	99.3

3.3. Chromatography

The total run-time for the XMP separation was 26.0 min (XMP retention time 12.0 min). A high quality separation of XMP was realized without underlying peaks at the same retention time by using the described chromatographic conditions, as exemplary demonstrated in Fig. 3a–c. The limit of detection (signal to noise ratio of 3) was 5 pmol at an injection volume of 10 μl for XMP. The concentration after incubation and deproteinization of the sample with an IMPDH enzyme activity of 3 nmol XMP/gHb/h was approximately three-fold higher, the limit of quantification (LOQ) therefore sufficient to identify patients even with a very low IMPDH enzyme activity.

3.4. Assay recovery and stability

The assay recover of XMP was approximately 100% as detailed in Table 1 and demonstrated a high intra- and inter-day stability ($\bar{x}_1 = 3.04 \pm 4\%$, $\bar{x}_2 = 5.11 \pm 3\%$, $\bar{x}_3 = 11.29 \pm 3.5$ nmol XMP/gHb/h and $\bar{x} = 3.08 \pm 4\%$ nmol XMP/gHb/h), respectively.

3.5. Determination of the IMPDH enzyme activity in ALL

The IMPDH enzyme activity in erythrocytes ranged wide within both, children with ALL ($n = 75$) and healthy adult controls ($n = 35$) as evident from the histograms (Fig. 4). Children with ALL had a mean enzyme activity of 10.42 ± 9.49 nmol/gHb/h (range: 0.8–42) whereas the mean enzyme activity was found to be significant lower (6.30 ± 3.51 nmol/gHb/h (range: 1.6–14.6)), $P < 0.03$.

4. Discussion

Quantitative measurements of the enzyme activities in purine metabolism have been recommended to ensure optimal thiopurine therapy for children with ALL [11,14,22,23]. In resent years, almost all researchers have focused on degradation metabolism by measuring TPMT enzyme activity because of the known polymorphism occurring in the TPMT gene, which is responsible for the tremendous differences of measurable activities of the TPMT enzyme [11,13,14,21,22]. So, before starting thiopurine therapy, children with ALL were classified as having low, intermediate or high TPMT enzyme activity. This designation was meant to avoid under treatment in patients

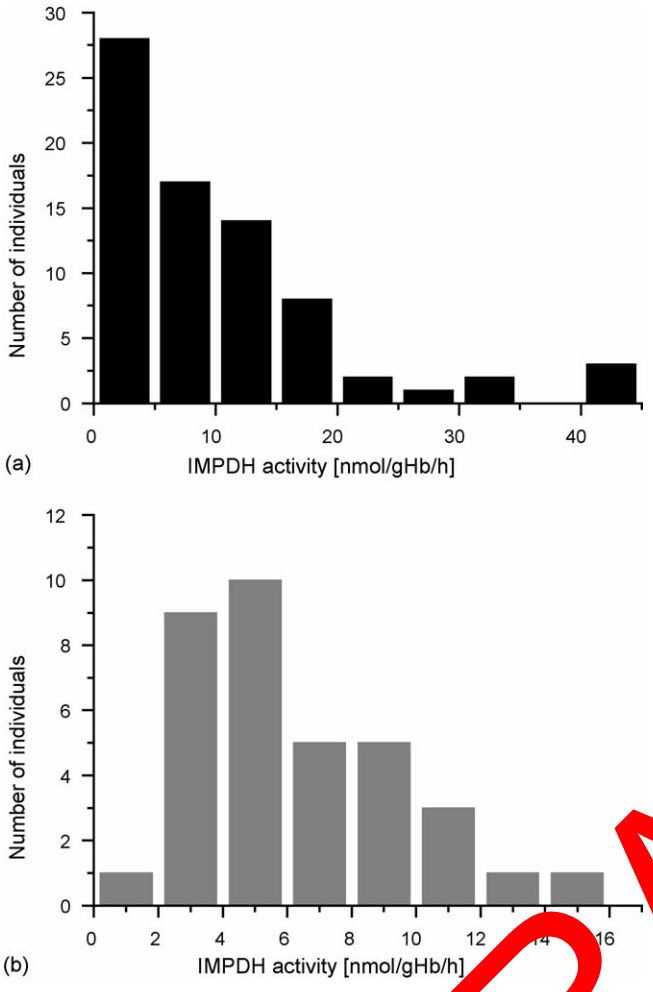

Fig. 4. IMPDH enzyme activity in erythrocytes. Distribution (histo…) IMPDH enzyme activity in 110 individuals, 75 children a…nosis of ALL before treatment (a) and 35 healthy adult vol…eers (b). The …PDH enzyme activity was determined as described and r… the wide range o… easurable enzyme activities found in both, childre… with AL… d healthy adul…

with high enzyme activit…s or severe complica…on in children with low activities w… were gi…n the standard drug regimen [4,14–16,23]. However, … IMPDH enz…me activity represents the rate-limiting …yme fo… the 6-thi…guanine nucleotide synthesis and is …refor… the criti… e…yme to process anticancer therapy w…h thiopu…nes [5,24]. Few authors have addressed this issue b… meas…ing th… IMPDH enzyme activity in children with ALL [17…]. More often, the IMPDH enzyme activity is measured in mo… uclear cells to monitor the immunosuppressive efficiency of m…cophenolate mofetil (MMF, CellCept®), a reversible inhibitor of the IMPDH enzyme, in transplant recipients [25,26]. To our knowledge only four studies have used erythrocytes, to determine the IMPDH enzyme activity so far [18,26–28]. Pehlke et al. and Lommen et al. investigated the IMPDH enzyme activity in patients deficient in the hypoxanthine guanine phosphriboyltransferase (Lesch–Nyhan syndrome) using a radioactive HPLC-based approach with contradictory results [27,28]. The same HPLC-based approach was used by Price et al. in 10 patients with ALL using bone marrow or lymphocytes from peripheral blood [17]. A non-radioactive assay using erythrocytes was first introduced by Montero who measured the IMPDH enzyme activity in a small series of patients with a mixed diagnoses of leukaemia and lymphomas [18]. However, Price detected remarkable differences in the IMPDH enzyme activity within a group of children with ALL, as well as between the group and normal controls, while Montero did not [17,18]. Whether these opposing findings are related to the sample size, assay method or source used to determine the IMPDH activity is unclear. The present investigation addresses these issues.

Erythrocytes represent the standa… source …o determine enzyme activities of the thiopuri… metabolism …n children with ALL [18,26–28]. We therefore …ablished a H…LC-based method to determine the IM…DH enzy…e activity …n erythrocytes from banked donor …ood. The assa… wa…hen used on erythrocytes from a lar… group o… patients w…h an ALL diagnosis.

The remarkab… features o… his assa… are the simple erythrocyte separatio… molysis and …s… conditions as well as a distinct seg…gation … the XMP fr…m the clear supernatant after precipitation by optima… IPLC conditions. The erythrocyte separati… and haemolysis wa… arried out by centrifugation following… wo washing cycles with 0.9% saline and a freeze-thawing cy…e. Thus, the a…ditional use of oxidation–reduction reagents lik…thioerythrit… (DTE)/DTT and mercaptoethanol as well as a gel f…ation f… the haemolysis could be avoided. This contradicts Montero who acknowledged its responsibility for a marked d…e in the resulting enzyme activity [17]. Optimal KCl and DTT concentrations are warranted, despite the requirement of using saturated substrate and co-substrate concentrations to obtain optimal assay conditions. Otherwise, a decrease of the measurable enzyme activity occurs. By using the described assay conditions, we have observed a proportional product building velocity dependent on the amount of haemolysate used, up to the maximal incubation time of 24 h. Montero, however, found a proportional product building velocity limited to 2.5 h by using an enzyme concentration twice the strength of ours by simultaneously lowering the determined substrate and co-substrate concentration [17]. The exact pH adjustment (pH 5.4) of the extracts seems to be most important in this context since a varying pH of 0.5 from the optimum results in overlapping peaks. A high-quality HPLC-based separation of XMP can be realized by simple $HClO_4$ precipitation followed by perchlorate precipitation (K_2HPO_4) with simultaneously pH adjustment. Thus the need for Montero's lavish extraction procedure with diethylether and a heating step, could be avoided [17,26].

Our IMPDH enzyme activity assay is impressive because of its high sensitivity and reproducibility, a 100% recovery rate and a good intra- and interday stability in comparison to previous reports [17,26]. The HPLC assay reported here was used to determine the IMPDH enzyme activity (at diagnosis) in a total of 75 untreated children with ALL and in 35 healthy adult volunteers. This is the first report, to our knowledge, investigating the IMPDH enzyme activity in erythrocytes in a large number of untreated patients with ALL. In direct comparison to Becher and co-worker and Price et al. our enzyme activity analysis demonstrated significantly higher values in patients with ALL

compared to healthy controls by using different methods and source to determine the IMPDH enzyme activity [17,29]. Further analyses are needed to clarify the clinical impact of the wide-ranging IMPDH enzyme activity found in children with ALL. Thus, the IMPDH enzyme activity in children with ALL has to be measured and correlated with the TPMT enzyme activity to estimate its influence on an optimal therapy with thiopurines and improve outcome in childhood ALL since the cause of treatment failure in 20% is largely unknown [30].

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