Original Article

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Determination of inosine 5'-monophosphate dehydrogenase activity by high performance liquid chromatography in comparison with normalization methods

MT. Nguyen[1,2,3], NT. Tran[1], M. Vincent[2,3], A. Capron[2,3], M. Mourad[4], P. Wallemacq[2,3]*

[1] Biochemistry Department, University of Medicine and Pharmacy, Ho Chi Minh city, Viet Nam

[2] Clinical Chemistry Department, Cliniques universitaires St Luc, Brussels, Belgium

[3] Louvain center for Toxicology and Applied Pharmacology, Université catholique de Louvain, UCL, Brussels, Belgium

[4] Surgery and abdominal transplantation department, Cliniques universitaires St Luc, Brussels, Belgium

Abstract

Mycophenolic acid (MPA) is a potent, selective and uncompetitive inhibitor of the inosine 5' -monophosphate dehydrogenase (IMPDH), which is the rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides being required during T and B lymphocyte proliferation. In this study, HPLC method was developed and validated to determine the IMPDH activity in human peripheral blood mononuclear cells (PBMCs) for the purpose of investigating the effect of MPAon lymphocytes. PBMCs were isolated from total blood samples. IMPDH activity in PBMCs was expressed according to two normalisation methods, namely as the ratio of produced xanthosine monophosphate (XMP) (micromoles) versus either adenosine monophosphate (AMP) (moles), or protein (mg) concentration per incubation time. IMPDH activities were assessed in 41 healthy volunteers. The total HPLC run time was 14 min. This method was linear in the range of 0.5-80 µmol/L for both AMP and XMP. Mean intra-assay and inter-assay precision and accuracy at quality control (QC) levels were > 94% for AMP and XMP. The recovery was > 75% for AMP and XMP. The lower limit of quantification of both AMP and XMP was 0.5 µmol/L. No interference was identified in the assay. Both IMPDH activity normalisation methods in our study displayed good and similar results, however, the protein normalisation method displayed slightly better reproducibility in our population cohort.

Keyword: inosine 5'-monophosphate dehydrogenase activity, mycophenolic acid, HPLC, peripheral blood mononuclear cells

1. INTRODUCTION

Immunosuppressive drugs are widely used in transplantation to prevent early acute rejection and to provide long-term effective rejection prophylaxis. Currently, various combinations of immunosuppressive agents are typically used both to minimize individual dosages and to take advantage of their synergistic effects.[1] Most drugs have a narrow therapeutic range, and empirical dosing can lead to either over-immunosuppression (i.e., side effects or infection) or under-immunosuppression (i.e., increased risk of rejection).[2] Moreover, a large inter- and intra-individual pharmacokinetic variability may justify the use of therapeutic drug monitoring (TDM). TDM has been used for decades assuming the blood drug concentration (drug exposure) is closely linked to drug efficacy. However, conventional TDM of immunosuppressive drugs, even though mandatory, has demonstrated some

***Corresponding author:** E-mail: pierre.wallemacq@uclouvain.be

limitation in predicting the pharmacological effects on immune cells. Indeed, similar blood concentrations may still result in different individual outcome. It could be expected that pharmacodynamic (PD) biomarkers monitoring better reflects the drug's biologic effects.[3-7]

Mycophenolic acid (MPA) is a potent, selective and uncompetitive reversible inhibitor of the inosine monophosphate dehydrogenase (IMPDH), which is the rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides being selectively required by T and B lymphocyte proliferation.[8-9] IMPDH catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent conversion of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP). Thus, the measurement of IMPDH catalytic activity in lymphocytes could serve as a tool to investigate the MPA effect on lymphocytes.[8-9] The aim of this study is, therefore, to develop and validate a high performance liquid chromatography method to determine the IMPDH activity in human and to compare different methods of normalisation.

2. MATERIALS AND METHODS

2.1. Reagents

High-performance liquid chromatography (HPLC) grade methanol was purchased from J.T. Baker (Deventer, Netherlands). Triethylammonium phosphate Buffer 1M (pH 3), Dulbecco's phosphate buffered saline, IMP, AMP, NAD^+, XMP and bovine serum albumin were obtained from Sigma Life Science and Sigma-Aldrich Chemicals (St.Louis, Switzerland; Steinheim, Germany). Leucosep tubes with Ficoll-Paque solution were obtained from Greiner Bio-One (Wemmel, Belgium). Potassium chloride, potassium carbonate and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany).

2.2. Patients and Samples

Heparinized blood was obtained from 41 healthy volunteers (25F/16M ranging from 21-58 yr). All subjects were given their informed consent. The local Ethics Committee has approved the blood sampling for this purpose. All samples were processed at room temperature within 12 hrs after collection.

2.3. Isolation of PBMCs from the whole blood

The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by a leucosep® tubes according to the manufacturer's protocol and to the procedure of Glander et al.[10] with minor changes. Lithium heparin blood samples (2.5 mL) were carefully poured into a leucosep® tube 12 mL and centrifuged for 20 min at 1200 g at room temperature without brakes. After discarding the plasma layer fraction, PBMCs were harvested and washed with 10 mL of PBS and subsequently centrifuged at 1000 g for 20 min at 4°C. The cell pellet was resuspended in 250 µL of water (HPLC grade, 4°C) and stored at -80°C. After thawing the frozen cell pellet, insoluble fragments were removed by centrifugation at 1200 g for 5 min. The supernatant lysate was used for enzymatic assay (100 µL) and protein (20 µL) determination. The protein concentration was determined according to the Lowry method.

2.4. IMPDH activity assay

The IMPDH activity in PBMCs was measured by HPLC. All samples were analysed in duplicate. 300 µL of reaction environment, containing 40 mM sodium dihydrogen phosphate pH 7.4, 100 mM potassium chloride, 0.5 mM IMP, and 0.5 mM NAD^+, were incubated at 37°C for 5 min. The kinetic enzymatic reaction was performed within 3 hrs after adding 100 µL supernatant lysate to the 300 µL of reaction environment. 50 µL of reaction mixture was removed sequentially to stop the enzymatic reaction and added into a 1.5 mL eppendorf containing 12.5 µL of 10% perchloric acid maintained on ice, at 0, 45, 90, 120, 150 and 180 min. Centrifugation of the eppendorfs was performed at 14,000 g at 4°C for 5 min in order to remove precipitated proteins. Subsequently, 45 µL of supernatant were neutralized with 4 µL of 1.5 M K_2CO_3 and then centrifuged again at 14,000 g at 4°C for 5 min. The supernatants were stored at -80°C until assay or immediately injected into the HPLC column to determine

the produced XMP and intracellular adenosine monophosphate (AMP). IMPDH activity in PBMCs was expressed as the ratio of produced XMP (µmol) versus either adenosine monophosphate (AMP) (mole) per incubation time (µmol XMP.s^{-1}.mol^{-1} AMP) or protein concentration per incubation time (nmol XMP.h^{-1}.mg^{-1} protein).

2.5. HPLC analysis

XMP and AMP separation was carried out using a Lichrocart C18 column (125 x 4 mm, 5 µm) maintained at 40°C on a HPLC Alliance 2695 instrument (Waters) equipped with a photodiode array detector set at 254 nm. The mobile phase consisted of a mixture (2:98, v:v) methanol and triethylammonium phosphate buffer (pH 6.5) set at a flow rate of 1 mL/min. The isocratic separation was performed during 6 min after injecting 10 µL of sample onto the column and then a gradient mode was used for 8 min to clean the column.

2.6. Method validation

8 concentrations of AMP and XMP ranging from 0.5 to 80 µmol/L were used for the calibration curve. Three concentrations of 0.75, 7.5 and 30 µmol/L AMP and XMP were used as quality controls and prepared separately. Calibrators were prepared following the procedure described by Glander et al.[9] The validation criteria included: linearity, intra- and inter-assay accuracy and imprecision, recovery, sensitivity and specificity according to the US Food and Drug Administration's Guidance for industry 2001.[11]

Imprecision is expressed as coefficient of variation (%); accuracy is expressed as percentage of closeness to the target value. Lower limit of quantification (LLOQ) of AMP and XMP was assessed including the criteria of reproducibility for replicate injections (coefficient of variation, 20%). The validated method was applied for 41 blood samples obtained from healthy volunteers in order to determine the normal range of IMPDH activity. The quantification of IMPDH activity was performed in triplicate on each blood sample. Enzyme activity was normalised based either on measured intracellular AMP concentrations obtained from the same HPLC method, or on cellular lysate protein concentrations.

Enzyme stability over time was checked with 3 aliquots of lysate samples stored at -80°C for a month, and the enzymatic samples maintained at 4°C into the autosampler for 48 hrs.

2.7. Statistical Analyses

Means and standard deviations (SDs) were calculated with Excel software 2010 (Microsoft). The JMP 9 software (*JMP* software, SAS Institute, Cary, NC, USA) was used to assess the correlation between IMPDH activities and MPA trough levels. Paired 2-tailed Student t-tests were used for the comparison of 2 means and 1-way analysis of variance when dealing with more than 2 means. Results were considered significant if $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Chromatographic Separation

The total HPLC run time was 14 min. XMP and AMP were eluted at 2.1 and 4.6 min, respectively, then HPLC column was washed for 8 min after. No interference has been identified in the assay (See Figure 1).

3.2. Calibration, Lower Limit of Quantification (LLOQ), Accuracy

Typical regression equations for calibration plots were Y = 4453X + 315.96 for AMP and Y = 3435.6X + 137.85 for XMP, where X is the concentration and Y is the peak area of the analyte. The calibration curve was linear in the range of 0.5-80 µmol/L for both AMP and XMP. The coefficient of determination (r^2) ranged from 0.998-1.000, and from 0.997-0.999, for AMP and XMP, respectively. Mean intra-assay and inter-assay precision levels were above 94% for AMP and XMP. The accuracy ranged from 95.49-108.90%, and from 94.22-115.19%, for AMP and XMP, respectively. The recovery was above 75% for AMP and XMP. The lower limit of quantification of both AMP and XMP was 0.5 µmol/L (See Table 1).

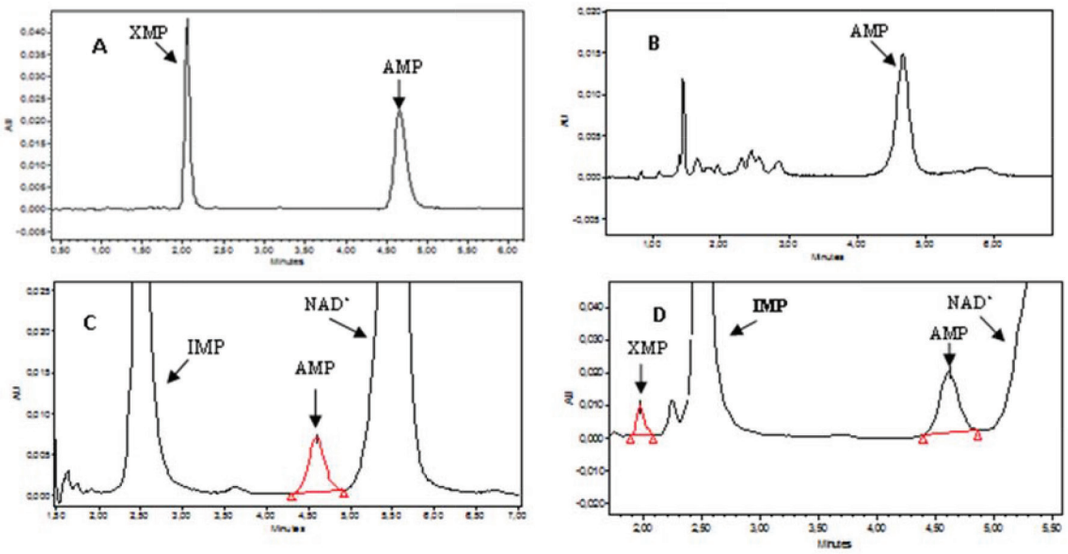

Figure 1. Representative chromatogram of AMP and XMP obtained from a calibrator sample at 50 µmol/L (A). Chromatogram of AMP determined from the enzymatic reaction samples at time 150 min without IMP substrate nor co-substrate NAD+ (B) and containing co-substrate NAD+ but not IMP substrate (C). Chromatogram of AMP and XMP determined from the enzymatic reaction samples involved IMP substrate and co-substrate NAD+ at time 150 min (D).

Table 1. Mean observed concentrations expresses as mean ± Standard Deviation (SD), mean intra-day and inter-day accuracy and precision for XMP and AMP calibrators at Lower Limit of Quantification (LLOQ) and three levels of Quality Control (QC samples)

	Mean observed concentrations ± SD (µmol/L)		Intra-day (n = 4) Accuracy/precision (%)		Inter-day (n = 4) Accuracy/precision	
	XMP	AMP	XMP	AMP	XMP	AMP
LLOQ	0.47 ± 0.03	0.57 ± 0.08	94.22/ 95.29	95.94/ 93.94	115.19/ 98.48	104.59/ 94.82
Low QC	0.77 ± 0.02	0.79 ± 0.04	102.36/ 95.08	97.65/ 98.89	106.39/ 95.36	108.90/ 94.98
Medium QC	7.41 ± 0.08	7.21 ± 0.029	98.96/ 96.82	96.16/ 96.37	95.19/ 94.02	96.15/ 96.16
High QC	30.57 ± 0.57	30.05 ± 0.05	101.91/ 98.84	101.95/ 98.52	100.98/ 95.01	100.16/ 95.97

3.3. Stability

No significant difference in IMPDH activity was found between initial lysate samples and samples at -80°C, and extracts maintained at 4°C into the autosampler for 48 hrs.

3.4. IMPDH Activity

XMP values exhibited a linear increase in the enzymatic reaction with the incubation time. AMP was present in PBMCs but AMP concentrations widely increased from 0 up to 180 min of incubation time (from 18.25 ± 3.87 µmol/L, CV (coefficient of variation) 21.23% to 62.93 ± 14.42 µmol/L, CV 22.91% , respectively).

Currently, there are two widely accepted manners to normalize enzyme activity, namely cell count, and even more frequently, protein concentration.[12] Glander P et al. have recently developed a method of IMPDH activity standardization based on the concentration of intracellular AMP[10], and the enzymatic activity of IMPDH was determined at 150 min. In the study of Glander et al., IMPDH activity in PBMCs was expressed as the ratio of produced XMP (µmol) versus either adenosine monophosphate (AMP) (mole) per incubation time (µmol XMP/s/mol AMP). The authors determined the enzymatic activity of IMPDH at 150 min of the enzymatic reaction because the AMP concentrations reach a plateau after 120 min of incubation time. Actually, we recognized that IMPDH activity normalized by AMP quantity continuously decreased from 120 min to 180 min of incubation time. On the contrary, IMPDH activity normalized by lysate protein concentration linearly increased during 180 min of enzymatic reaction. After normalisation, the IMPDH activity at each determination time of enzymatic kinetic reaction displayed a slightly better CV% when normalised by protein concentration versus by AMP. Among the healthy volunteers, IMPDH results exhibited an inter-individual variability, for example the IMPDH activity determined at 150 min ranged from 12.63 to 33.76 µmol/s/mol AMP (or from 4.11 to 10.34 nmol/h/mg protein). Neither age nor gender appeared affecting the IMPDH activity.

In our study, we assessed two IMPDH activity normalisation methods based on AMP concentration and on protein concentration for healthy volunteers and patients groups. We found that AMP concentrations in cell lysate significantly increased from 0 up to 180 min of incubation time, whereas IMPDH activity normalized by lysate protein concentration was stable during 180 min of the enzymatic reaction. Moreover, we could stop the enzymatic reaction before 150 min if IMPDH activity was normalized by protein concentration (See Table 2 and Figure 2).

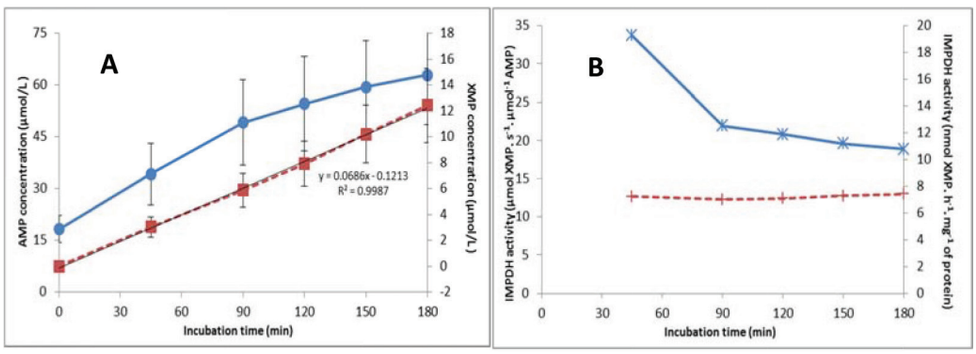

Figure 2. The produced XMP (square) and intracellular AMP (circle) concentrations determined within 180 min of enzymatic reaction (A) and IMPDH activity normalized either by intracellular AMP concentration (star) or by cell lysate protein concentration (cross) from 41 healthy volunteers samples (B)

Table 2. IMPDH activity determination within 180 min of the incubation time from 41 different healthy volunteer samples

	Cell lysate	Incubation time (min)				
	0	45	90	120	150	180
Healthy volunteers (n = 41)						
Protein concentration (mg/ml)	(2.10-4.90)					
	(2.10-4.90)					
CV%	20.38					
XMP concentration (µmol/L)		3.02 ± 0.79 (1.62-4.88)	5.86 ± 1.30 (3.36-9.06)	7.89 ± 1.74 (4.29-11.72)	10.19 ± 2.24 (6.43-15.88)	12.43 ± 2.87 (7.37-20.08)
CV%		26.32	22.22	22.08	22.03	23.10
AMP concentration (µmol/L)	18.25 ± 3.87 (8.72-25.41)	34.16 ± 8.95 (19.94-58.93)	49.11 ± 12.31 (28.05-82.74)	54.52 ± 13.65 (32.62-88.23)	59.37 ± 13.35 (35.08-97.64)	62.93 ± 14.42 (33.20-104.81)
CV%	21.23	26.20	25.07	25.03	22.49	22.91
IMPDH activity (µmol XMP. s^{-1}. μmol^{-1} AMP)		33.68 ± 9.32 (20.24-64.28)	22.89 ± 5.59 (13.99-37.54)	20.83 ± 5.30 (11.53-35.05)	19.62 ± 4.75 (12.63-33.76)	18.85 ± 4.74 (11.60-30.29)
CV%		27.67	24.15	25.46	24.22	25.17
IMPDH activity (nmol XMP. h^{-1}. mg^{-1} of protein)		7.21 ± 1.99 (4.26-10.94)	7.01 ± 1.63 (4.21-10.81)	7.09 ± 1.72 (3.87-10.80)	7.28 ± 1.51 (4.11-10.34)	7.41 ± 1.65 (3.92-10.89)
CV%		27.58	23.29	24.29	20.74	22.37

Both IMPDH activity normalisation methods could display good results, however, the protein normalisation method displayed better reproducibility in our population cohort.

4. CONCLUSIONS

Therapeutic drug monitoring (TDM) of immunosuppressive drugs could reduce the rejection rates and limit the occurrence of side effects.[4] In clinical practice, evaluation of drug concentrations to adjust drug dosing is already routinely established but may not necessarily predict the pharmacologic effects on immune cells. Moreover, resulting effect of combined immunosuppressive therapies, polymorphism of drug targets, and overall inter-individual differences of organ donors and recipients may limit the efficiency of regular TDM in predicting clinical efficacy.[13] On the contrary, pharmacodynamic (PD) monitoring may assess the efficacy of immunosuppressive drug therapy. So measuring biomarkers in addition to drug concentrations may be interesting and complementary to predict clinical outcome in an individual patient. IMPDH activity is a specific pharmacodynamic parameter of mycophenolic acid activity. It is necessary to develop an assay method less labor-intensive, more robust, and useful for determining IMPDH activity in routine. Several studies attempted to identify a target range for inosin-5'-monophosphate dehydrogenase activity in maintenance therapy with tacrolimus in association with mycophenolate mofetil.[14-16] We described in

this study a robust, inexpensive, rapid and simple method suitable for the determination of IMPDH activity in peripheral blood mononuclear cells.

5. ACKNOWLEDGMENTS

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