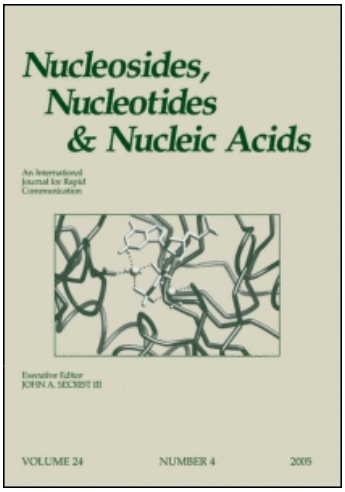

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HPLC/TANDEM ION TRAP MASS DETECTOR METHODS FOR DETERMINATION OF INOSINE MONOPHOSPHATE DEHYDROGENASE (IMPDH) AND THIOPURINE METHYLTRANSFERASE (TPMT)

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□ *The efficiency of Mycophenolate mofetil (MMF) and Azathioprine (AZA) as immunosuppressive agents depends on the activity of 2 enzymes, inosine monophosphate dehydrogenase (IMPDH) and thiopurine methyltransferase (TPMT) respectively. We present preliminary evaluation of nonradioactive methods that apply HPLC with ion-trap mass detection to measure the activities of IMPDH in peripheral blood mononuclear cells (PBMC) and TPMT in the erythrocytes (RBC). We found IMPDH activity of 0.9 ± 0.2 nmol/hour/10^6 PBMC and TPMT activity of 19.9 ± 4.7 nmol/hour/ml RBC in healthy subjects. These methods, following its further validation, could be useful for monitoring the activity in a clinical and experimental setting.*

Keywords Mycophenolate mofetil; Azathioprine; Inosine monophosphate dehydrogenase; Thiopurine methyltransferase; LC/MS; HPLC

INTRODUCTION

Clinical effects and adverse reactions of AZA or MMF are known to be dependent on variations in gene loci encoding the enzymes of nucleotide metabolism. In the case of Azathioprine inherited differences in TPMT activity is an important factor in the wide interindividual variations observed in the clinical response to thiopurine therapy. TPMT activity is known to be

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under control of a common genetic polymorphism.[1,2] In the case of MMF metabolism the two IMPDH enzymes Type I and Type II catalyze the conversion of inosine-5′-monophosphate to xanthosine-5′-monophosphate. High basal or induced expression of IMPDH would be predicted to result in poor MMF efficacy at normal doses.[3] The standard spectrophotometric method of measuring IMPDH activity requires a large quantity of sample and the most commonly used method for TPMT monitoring uses radioisotopes.[4] The mass spectrometric methods described so far, have been based on application of the expensive triple-quadrupole mass detectors. Our aim was to develop methods that allow the measurement of TPMT and IMPDH activities with the use of the more widely available ion-trap mass detector.

MATERIALS AND METHODS

TPMT Assay and LC/MS Conditions

Blood from 11 healthy volunteers was collected in heparinized tubes and centrifuged to isolate red blood cells (RBCs). Erythrocytes were lysed and gently rotated with Chelex resin. Aliquots of lysed erythrocytes supernatant were incubated with: 0.75 M KH_2PO_4 pH 7.5, 12 μM S-adenosyl-methionine, 48 μM allopurinol, 1 mM dithiothreitol, and 3.8 mM 6-MP for 1 hour at 37°C and stopped by adding zinc sulphate/methanol solution containing the internal standard: 10 μM 2-Chloro-Adenosine. A 3 μm Hypersil C18-BDS column 150 × 2.0 mm was used for the separation of substrate, product, and internal standard. HPLC buffer A was 0.05% formic acid and buffer B (100% acetonitrile) were run at 0.2 ml/min. The mass detector (LCQ Deca XP) data were acquired in full mass range ($m/z = 70–700$) and in single ion monitoring mode for 6-MMP ($m/z = 167.2$). Ion chromatograms were then extracted for substrate and internal standard from full MS mode and for product from single ion monitoring mode.

IMPDH Assay and LC/MS Conditions

Peripheral blood mononuclear cells (PBMCs) were isolated from 7 healthy volunteers by centrifuging EDTA treated blood layered on lymphoprep. After washing, PBMCs were counted before lysis by freeze thawing followed by sonication. Aliquots of PBMCs were incubated with: 21 mM NaH_2PO_4/54 mM KCl pH 7.4, 0.27 mM NAD, and 0.27 mM IMP. The mixture was incubated for 2 hours at 37°C. The reaction was stopped with 2.5 M perchloric acid and neutralized. A 3 μm Develosil RP-AQ column 150 × 2.0 mm was used for the separation of substrate, product and internal standard (10 μM 2-Chloro-Adenosine). HPLC buffer A was 10 mM ammonium acetate/2 mM acetic acid and buffer B was acetonitrile. MS

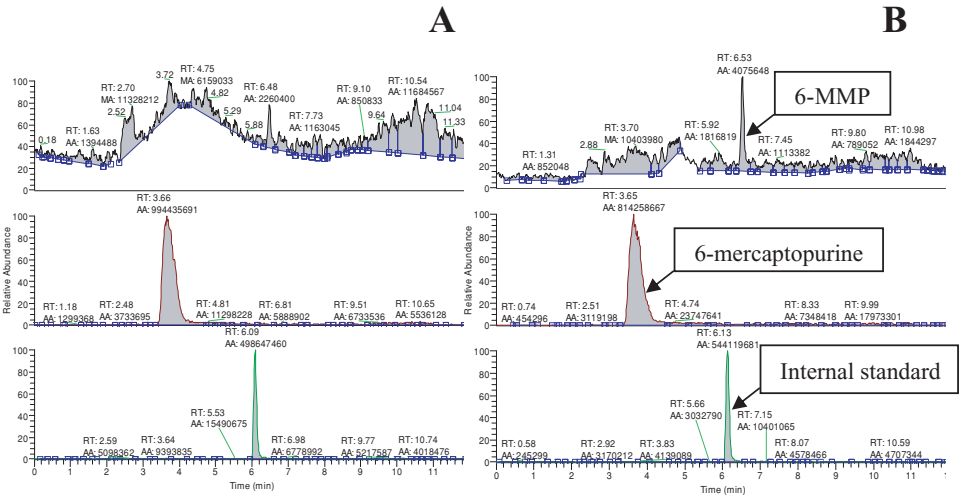

FIGURE 1 Ion chromatograms showing from the top: product (6-MMP, RT 6.53) (upper panel), substrate (6-mercaptopurine, RT 3.65) (middle panel), and internal standard (2-Chloroadenosine, RT 6.08) (lower panel) in the blank without incubation (A) and after 1 hour of incubation (B).

data were acquired in positive ion mode with fragmentation (MS^2). In MS^2 mode, fragment ions for XMP (product) were detected at *m/z* 153 while IMP (substrate) fragments were detected at *m/z* 137.

RESULTS

Figures 1A and 1B presents TPMT activity determination using LC/MS and demonstrates detection of the substrate, product and internal standard. Erythrocyte lysate was incubated for 1 hour compared to the blank where no detectable 6-MMP can be observed.

Figures 2A and 2B presents determination of IMPDH activity. The product XMP was clearly visible after incubation, while no signal was detected in the blank sample. Calculated data for the activity determination are presented in Table 1, showing the average and range of values in a group of healthy volunteers.

TABLE 1 Enzyme Activities of TPMT and IMPDH in Healthy Subjects

	n	Average activity ± SEM	Range
TPMT	11	19.9 ± 4.7 nmol/hr/ml erythrocytes	15.4–29.2
IMPDH	7	0.9 ± 0.2 nmol/hr/10^6 PBMCs	0.2–3.1

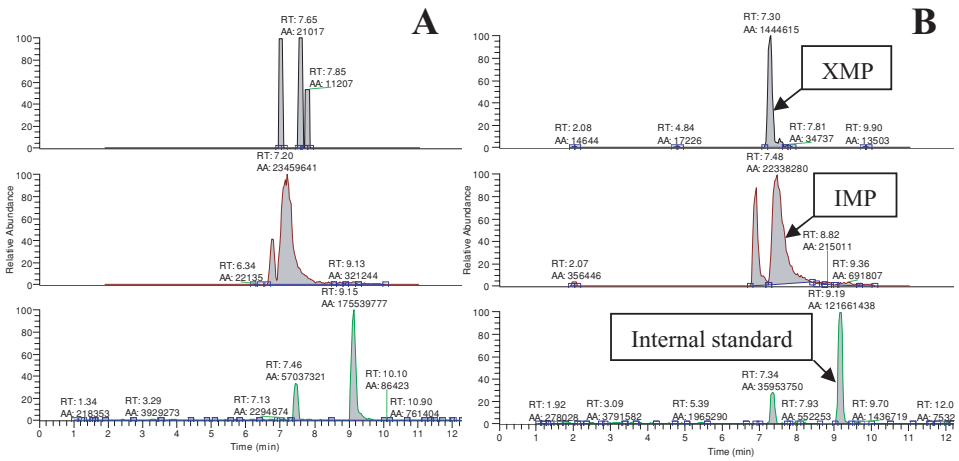

FIGURE 2 Ion chromatograms showing from the top: 1—product, XMP detected in MS^2 fragmentation mode (upper panel), 2—substrate, IMP detected in MS^2 fragmentation mode (middle panel), and internal standard (2-Chloroadenosine) extracted from full MS mode (lower panel). (A) blank without incubation, (B) after 2 hour of incubation.

DISCUSSION

Our preliminary assessment of the feasibility of ion-trap LC/MS for determination of the TPMT and IMPDH activities highlights the potential suitability of this procedure for routine and experimental applications. This could be superior to standard radioisotopic and HPLC methods available for this analysis: smaller sample size necessary for analysis, more accurate identification of the substrates and products, avoiding the use of radioactive isotopes. Compared with other mass detection techniques it is important to indicate that ion-trap detectors are less expensive than triple quadrupole detectors. The methods described in this study require full validation, but they have the potential to be used routinely in the clinical setting due to its simplicity.

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