increased glial cell proliferation and S100B production. These associations also appear to be consistent with the hypothesis that, as a cytokine, S100B exerts a neurotrophic role (1), although more extensive studies of the relationships of S100B with other brain constituents will be needed to support this possibility. Previous investigations have reported that amniotic fluid is devoid of detectable S100B in physiological conditions, whereas detectable concentrations can be observed in anencephalic fetuses (9). The reason for the discrepancy between these findings and ours of low but measurable S100B concentrations in healthy fetuses is probably attributable to the different limits of detection of the methods used (0.2 μg/L in our study vs 1.5 μg/L for the method used in the previous study). The present data provide reference values for S100B in amniotic fluid during the second trimester of pregnancy, which could constitute a useful tool for the further study of pathological conditions of the nervous system in the early stages of pregnancy. In this respect, the source of a large part of S100B present in the amniotic fluid is probably the fetal nervous system, where the protein has been shown to be present at the ages investigated in the present study, although not at mature concentrations (13–16). On the other hand, it is possible that S100B could also be released, at least in part, from other sites in which it is concentrated, such as adipose tissue, although data on the presence of the protein in adipose tissue at this age are inconclusive. Finally, the possibility that S100B is released from placental tissue as a trophic factor should be taken into account, although its presence in the placenta has not been documented. In any case, the present findings offer preliminary data supporting further investigation of S100B dynamics in vivo, with special reference to a possible role of the protein in fetal brain maturation.

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References


Phenotype Determination of Thiopurine Methyltransferase in Erythrocytes by HPLC, Roselyne Boulieu,1,2 Martine Sauvait,2 Thierry Dervieux,2,3 and Jean-François Mercereaux1 (1 Université Claude Bernard Lyon 1, Département de Pharmacie Clinique, de Pharmacocinétique, et d’Evaluation du Médicament, 8 avenue Rockefeller, 69375 Lyon Cedex 08, France; 2 Hôpital Neuro-Cardiologique, Service Pharmaceutique, 59 boulevard Pinel, 69394 Lyon Cedex 03, France; 3 St. Jude Children’s Research Hospital, 332 N. Lauderale St., Memphis, TN 38101; 4 Hôpital Cardiologique, Service de Bronchopneumologie, 59 boulevard Pinel, 69394 Lyon Cedex 03, France; * author for correspondence: fax 33-04-72-35-73-31, e-mail roselyne.boulieu@chu-lyon.fr)

Thiopurine methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of thiopurine drugs, which are used in cancer chemotherapy and as immunosuppressive agents (1). TPMT activity is controlled by a common genetic polymorphism that contributes to interindividual variability in drug response and, consequently, to implications for thiopurine therapeutic efficacy and toxicity (2). Severe myelosuppression has been reported for TPMT-deficient patients treated with standard doses of thiopurines (3–5), and high TPMT activity has been associated with the rejection of transplanted organs (6). Because of the clinical significance of the TPMT genetic polymorphism, determination of the TPMT phenotype in red blood cells is routinely performed to optimize and individualize thiopurine treatment (5). Variant alleles of the TPMT gene have been characterized and associated with low TPMT activity.
Recently, Spire-Vayron de la Moureyre et al. (9) reported that genotypic analysis of TPMT allows the correct determination of metabolic capacity for 87% of individuals. A lower correlation was found for individuals with TPMT activity that was close to the antimode value. Thus, phenotypic analysis may be useful and could be performed concomitantly with genotyping tests. TPMT activity has classically been measured using a radiochemical assay (10). Few HPLC methods using nonradiolabeled calibrators with liquid-liquid extraction (11, 12) or solid-phase extraction (13) have been reported. Here, we report a reversed-phase HPLC method that uses a simple and rapid treatment procedure for the determination of TPMT activity in red blood cells.

Blood samples (5 mL) collected into lithium heparin tubes were centrifuged without delay at 1000 g for 10 min at 4 °C. Red blood cells were washed according to a previous protocol (13). The supernatant was stored at −80 °C. 6-Mercaptopurine (6-MP; 10 μL; final concentration, 4 mmol/L) was added to 300 μL of red cell lysates containing 100 μL of 0.15 mol/L potassium phosphate (pH 7.3) and preincubated for 3 min at 37 °C. The reaction was started by adding 30 μL of a mixture of S-adenosyl-l-methionine (SAM), toluene sulfonate salt, and dithiothreitol (final concentration of SAM, 25 μmol/L; final concentration of dithiothreitol, 1 mmol/L). The tubes were incubated for 1 h at 37 °C, and the reaction was stopped by heating for 3 min at 120 °C. After cooling, the tubes were centrifuged at 2000 g for 15 min at 15 °C, and an aliquot of the supernatant was analyzed by HPLC.

The methyl 6-MP (Me6-MP) formed was measured using a modification of the HPLC procedure described previously (14). Briefly, Me6-MP was analyzed on a Purospher RP 18-e column (Merck) with a linear gradient elution mode with 0.02 mol/L potassium dihydrogen phosphate (pH 3.5) and a mixture of 0.02 mol/L potassium dihydrogen phosphate (pH 3.5) and methanol (50:50 by volume). The methanol gradient was 0–35% over a period of 10 min. The flow rate was 1.2 mL/min, and Me6-MP was detected at 291 nm using a photodiode array detector. All analyses were performed at ambient temperature. Peak identity was confirmed through library matching by comparison of the unknown peaks with reference spectra.

The influence of 6-MP and SAM concentrations on TPMT activity was assessed using eight concentrations of 6-MP (0–7.5 mmol/L) at a constant SAM concentration of 25 μmol/L and eight concentrations of SAM (0–50.0 μmol/L) at a constant 6-MP concentration of 4 mmol/L. One unit of TPMT activity represents the formation of 1 nmol of Me6-MP per hour per milliliter of packed red cells at 37 °C. Results were normalized on the basis of the hematocrit. The apparent Michaelis–Menten constants (Kms) were estimated from six experiments using the Lineweaver–Burk plot.

The chromatogram of the red blood cell lysate from a subject is shown in Fig. 1A. 6-MP and Me6-MP were eluted at 6.2 and 13.1 min, respectively, with a total run time of 25 min. The mean analytical recoveries were 89.9% and 88.6% at concentrations of 25 and 150 μg/L, respectively. The calibration curve was linear over Me6-MP concentrations of 7.5–250 μg/L with a correlation coefficient >0.998. The quantification limit was 7.5 μg/L of packed cells with a CV <5% for a 300-μL sample volume. Intraassay and interassay CVs were <5.5% for replicate analyses of the red blood cell lysate supplemented with Me6-MP at three different concentrations: 10, 50, and 150 μg/L. Moreover, a quality-control lysate from volunteers in which TPMT activity (mean, 23.3 nmol/h per mL of packed cells; CV = 7.1%) was determined from replicate analyses during 8 months was included in each run.

In the conditions used, the formation of Me6-MP was
linear with respect to a lysate volume of 0–300 µL ($r^2 = 0.997$). The influence of the substrate concentration on TPMT activity is shown in Fig. 1, B and C. $K_{m,s}$ were 227 and 4.9 µmol/L for 6-MP and SAM, respectively, and the maximum velocities ($V_{max}$) were 28.1 and 24.9 nmol/h per mL of packed cells. These values are similar to those reported previously using a radiochemical assay (10, 15).

The TPMT activity determined in a population of Caucasian subjects was 10.4–41.7 nmol/h per mL of packed cells with a mean value of 28.9 nmol/h per mL of packed cells. These preliminary data are in close agreement with the results reported previously in an adult Caucasian European population (9, 13). One subject (2.4%) had TPMT in the intermediate range, 40 (97.6%) subjects had high TPMT activity, and no patient had low or undetectable TPMT activity. Although the number of subjects was small, our preliminary results were similar to the gaussian distribution reported recently in a European population (9, 12). In the method presented, according to Szumlanski et al. (15), the chelation step was omitted to reduce the time of analysis and to simplify the assay. Likewise, allopurinol was not added to the incubation mixture because of the absence of the enzyme xanthine oxidase in the erythrocytes. The simple and rapid sample treatment procedure described allows one to simultaneously stop the enzymatic reaction and obtain a clean extract that can be analyzed directly by HPLC. This procedure avoids the use of acid solutions that may induce potential degradation of thiopurine nucleotides even at ambient temperature (data not shown), and it avoids the time-consuming extraction step.

In conclusion, we believe the thiopurine methyltransferase assay described is rapid and reliable because of the lack of laborious liquid-liquid or solid-phase extraction. We also believe that this method could be implemented easily in the clinical laboratory for the phenotypic analysis of TPMT in patients scheduled for thiopurine therapy and could help optimize and individualize thiopurine treatment.

References


Detection of Nucleotide c985 A→G Mutation of Medium-Chain Acyl-CoA Dehydrogenase Gene by Real-Time PCR, Luis M. Real, Antonio J. Gayoso, Mercedes Olivera, Antonio Cariz, Agustin Ruiz, and Fidel Gayoso (1 Servicio de Bioquímica, Hospital Universitario Virgen del Rocío, Avda/Mansuelo Suyot s/n, 41013 Seville, Spain; 2 Biomedal, CIB Pabellón de Italia, C/Isaac Newton s/n, 41092 Seville, Spain; *author for correspondence: fax 34-954081279, e-mail liban@inicia.es)

Medium chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common hereditary defect of fatty acid oxidation in humans. This deficiency is an autosomal recessive disorder clinically characterized by episodic hypoglycemia, encephalopathy, apnea, and sudden death among children (1). A single A-to-G nucleotide transition at position 985 (nt c985 A→G) of the MCAD gene represents >81% of alleles causing MCAD deficiency (2). The frequency of this allele variant exhibits considerable geographical variation with a high prevalence in Northern Europeans (3).

PCR-based technologies are now widely used for the identification of the nt c985 A→G mutation for the MCAD deficiency (4, 5); however, they involve multiple steps and are time-consuming.

We used real-time PCR amplification coupled to fluorescence resonance energy transfer and melting curve analysis (6) to detect nt c985 A→G mutation of the MCAD gene using the single-step LightCycler technology.

In this study, we used genomic DNA isolated from EDTA blood from individuals who had been typed previously by PCR-restriction fragment length polymorphism analysis as was described by Matsubara et al. (7). DNA was isolated using the High Pure PCR Template Preparation reagent set (Roche Diagnostics) according to the manufacturer’s instructions. PCR was performed in a