ELSEVIER

Journal of Chromatography B, 727 (1999) 235–239

JOURNAL OF CHROMATOGRAPHY B

Short communication

Thiopurine methyl transferase activity: new extraction conditions for high-performance liquid chromatographic assay

C. Ganiere-Monteil*, A. Pineau, M.F. Kergueris, C. Azoulay, M. Bourin

Laboratoire de Pharmacologie Clinique, Institut de Biologie, Centre Hospitalier Universitaire, 9 Quai Moncousu, 44093 Nantes Cedex 1, France

Received 24 November 1998; received in revised form 11 February 1999; accepted 22 February 1999

Abstract

A new liquid–liquid extraction is described for thiopurine methyl transferase (TPMT, EC 2.1.1.67) activity determination: the use of a pH 9.5 NH_4Cl buffer solution, before adding the solvent mixture, allows more rapid extraction, avoiding a centrifugation step, and reduces the global cost of analysis. After the extraction step, 6-methylmercaptopurine, synthesised during the enzymatic reaction, is determined by a liquid chromatographic assay. Analytical performance of the assay was tested on spiked erythrocyte lysates. The linear concentration range was 5–250 ng ml^{-1} ($r \geq 0.997$, slope=1.497, intercept=−0.367). The recoveries were 82.8, 89.9 and 82.2% for 75, 125 and 225 ng ml^{-1}, respectively. The coefficients of variation were ≤6.1% for within-day assay ($n=6$) and ≤9.5% for between-day assay precision ($n=6$; 14 days). TPMT activity was determined in a French adult Caucasian population ($n=70$). The results ranged from 7.8 to 27.8 nmol h^{-1} ml^{-1} packed red blood cells and the frequency distribution histogram is similar to that previously published.

Keywords: Enzymes; Thiopurine methyl transferase; 6-Methylmercaptopurine

1. Introduction

Thiopurine methyltransferase (TPMT, EC 2.1.1.67) is a cytosolic enzyme involved in S-methylation of aromatic and heterocyclic sulphydryl compounds, such as thiopurine drugs 6-mercaptopurine and 6-thioguanine. In vivo, 6-mercaptopurine (6-MP) is converted to 6-methylmercaptopurine (6-MMP) by TPMT, S-adenosyl-L-methionine (SAM) being the methyl group donor. This catabolic pathway is one of the three competing pathways of 6-MP metabolism.

A genetic polymorphism is responsible for the wide interindividual differences observed in TPMT activity [1]. The frequency distribution of TPMT activity in large population studies is trimodal: 1 in 300 subjects have undetectable activity, 11% have intermediate activity and the remainder inherits high enzyme activity. It is important to determine TPMT activity in patients treated with azathioprine (organ transplant recipients) and 6-MP (leukaemic patients) [2], particularly to identify patients with undetectable activity.

TPMT activity has been firstly measured by a radiochemical assay [3]. More recently, an HPLC method has been published [4], later modified [5]. Both methods are based on the in vitro conversion of 6-MP to 6-MMP, using SAM as the methyl donor. In

*Corresponding author. Tel.: +33-2-40084095; +33-2-40084097.

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00083-3

the HPLC assay, 6-MMP synthesised during the enzymatic reaction, is extracted by a liquid–liquid extraction and measured by UV absorbance. This article describes a new liquid–liquid extraction, which reduces duration and cost of the analysis.

2. Experimental

2.1. Reagents and chemicals

6-MMP was supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). NH_4Cl, dithiotreitol, 2-propanol were obtained from Prolabo (Paris, France). Dimethylsulfoxide (DMSO), methanol, *o*-phosphoric acid and triethylamine were purchased from Merck (Darmstadt, Germany). Acetonitrile were obtained from SDS (Peypin, France) and dichloromethane from Mallinckrodt Baker (Deventer, The Netherlands). Acetonitrile was of HPLC grade, all other chemicals were of analytical grade.

A stock solution of 6-MMP (500 mg l^{-1}) was prepared in DMSO–methanol (1:1, v/v). A pH 9.5 buffer solution was obtained with a saturated NH_4Cl solution, diluted 25% with deionized water and adjusted to the desired pH by addition of 25%-diluted ammonia solution. Standard curves of 6-MMP (0–250 ng ml^{-1}) were prepared from appropriate dilutions in erythrocyte lysate.

2.2. Samples

Preparation of erythrocyte lysates is identical to that described in the initial assay [3]. Incubation conditions are similar to those previously described [4], except that all volumes are multiplied by 3 to improve the sensitivity (600 μl of erythrocyte lysate).

For the extraction step, to each tube, were added 1.0 ml of the NH_4Cl buffer (pH 9.5) and 5.0 ml of the extracting solvent (dichloromethane–2-propanol, 80:20, v/v). The tubes were shaken for 15 min, then centrifuged at 2800 *g* for 10 min at +4°C. The upper layer was discarded, and 3.5 ml of the organic phase were removed and evaporated to dryness under nitrogen. The residue was dissolved in 150 μl of the mobile phase and 50 μl were injected into the chromatograph. A 50-μl volume of acetonitrile was injected between two samples.

2.3. Chromatography

The analytical column was a Sulpeco LC-18 5 μm (250×4.6 mm I.D.). The mobile phase consisted of water–acetonitrile–triethylamine (91.7:8:0.03, v/v) and dithiotreitol 0.1 g l^{-1}. It was adjusted to pH 3.2 with *o*-phosphoric acid and filtered. The flow-rate was 1.0 ml min^{-1}. The detection wavelength was 290 nm.

3. Results and discussion

The chromatograms (Fig. 1) show a good resolution of chromatographic peaks. The 6-MMP retention time was 15.9 min and the total run time was 20 min. Acetonitrile injected into the chromatograph between each sample (run time=7 min) allowed elution of all products before the next injection.

The linearity was assessed from 5 to 250 ng ml^{-1}. The between-day variability of the regression line was studied during 8 days. The composite regression equation was $y=1.497x-0.367$ (y=6-MMP concentration, x=peak area). The coefficients r were all ≥ 0.997. The homogeneity of the results was demonstrated by the standard deviation of the slope 0.075. The standard deviation of the intercept was 2.118.

The precision was studied by adding 6-MMP to erythrocyte lysate at three different concentrations (75, 125 and 225 ng ml^{-1}). Blank values were subtracted. Lysates were analysed six times in a single run (within-day precision) and six times in separate runs over a period of two weeks (between-day precision). The method showed good precision with coefficients of variation (C.V.) of 2.7–7.3% and 4.1–9.5%, respectively, for within-day and between-day assay (Table 1).

During the extraction step, NH_4Cl buffer behaved as a haemoglobin precipitating agent. Consequently, after stirring and centrifugation, the solvent mixture remained transparent and colourless, contrary to the method published by Medard et al. [5]. This new extraction method does not alter the analytical recoveries which were 82.8, 89.9 and 82.2%, respec-

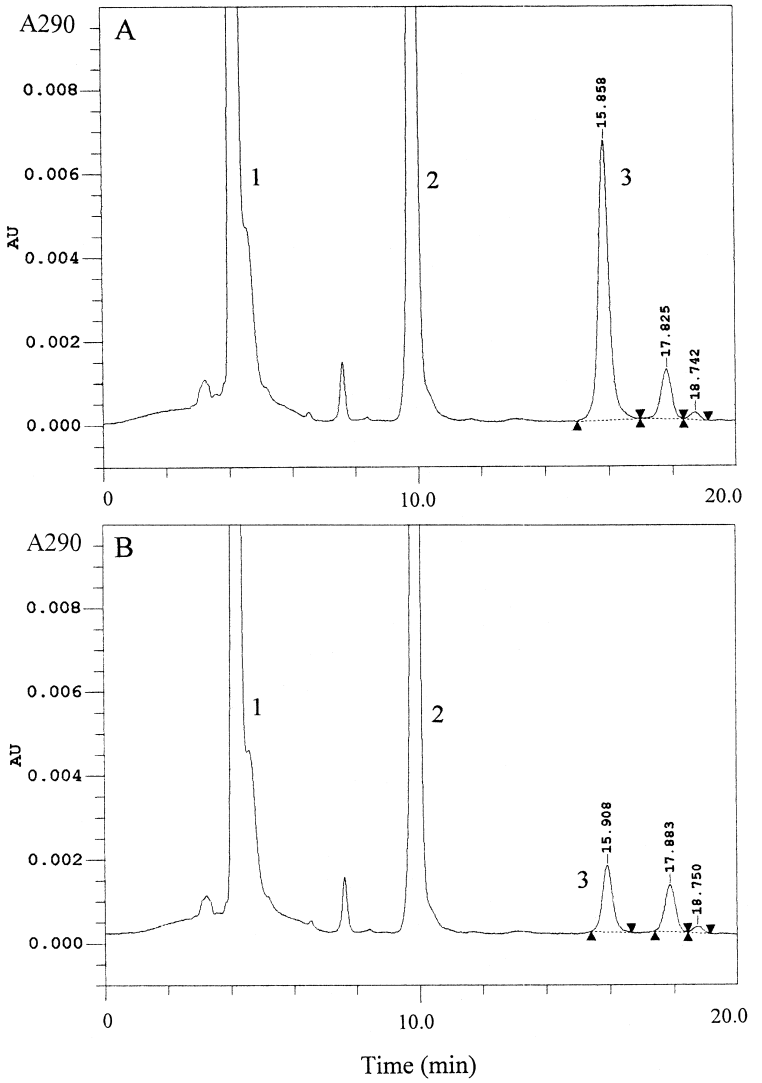

Fig. 1. Chromatograms of an erythrocyte lysate of an adult from the reference population. (A) incubation with SAM; (B) incubation without SAM. Peaks: 1=6-MP; 2=dithiotreitol; 3=6-MMP. 6-MMP was formed at the concentration of 182.8 ng ml^{-1} with a corresponding TPMT activity of 26.3 nmol h^{-1} ml^{-1} PRBC.

tively, for concentrations of 75, 125 and 225 ng ml^{-1}. Moreover, the haemoglobin precipitation avoids a step of centrifugation of the mobile phase which dissolves the residue after evaporation. This centrifugation was realised in centrifuge tube filters for low volumes which are expensive. This new extraction method is less onerous and time saving. The notion of economy of time is important for an assay which still remains very long: 1 h of gentle rotation of lysates with Chelex 100 resin and 1 h of enzymatic reaction incubation precede the extraction step.

The limit of detection, corresponding to the concentration that produces a peak area approximately three-times the baseline noise, was 5 ng ml^{-1}. The limit of quantification, defined as the lowest con-

Table 1
6-Methylmercaptopurine assay precision

Erythrocyte lysate level (ng ml^{-1})(mean±S.D.)	C.V. (%)
Within-day assay (n=6)	
77.2±5.6	7.3
127.2±7.8	6.1
231.0±6.3	2.7
Between-day assay (n=6, 14 days)	
75.6±4.5	6.0
123.5±11.7	9.5
228.6±9.4	4.1

centration analysed with acceptable accuracy (mean error in per cent ≤10%) and precision (C.V.≤10%) was 10 ng ml^{-1}.

The stability of the samples after extraction was studied during 32 h at room temperature: after extraction of spiked erythrocyte lysates, the mobile phase in which the residue was dissolved, was pooled and a chromatogram was recorded every hour. The stability was demonstrated during 30 h with a C.V. of the peak area of 0.3% (Fig. 2). This long stability is important because of the long chromatogram run time (2 chromatograms per hour) and the necessity to measure the blank value for each patient.

Table 2
Thiopurine methyltransferase activity (nmol h^{-1} ml^{-1} PRBC) in adult reference Caucasians

Population from	Number	Mean	Range	Ref.
USA	298	12.8	0–19	[1]
Norway	50	13.1	6.7–19.7	[6]
France	300	19.3	4.7–35.3	[4]
France	303	15.4	2–40	[7]
France	70	18.2	7.8–27.8	Our study

A quality control prepared by spiking erythrocyte lysate was included in each assay. The inter-assay variation was 6.6% (n=6). The 6-MMP stock standard solution at 500 mg l^{-1} in DMSO–methanol remained stable for 6 months at −20°C.

TPMT activity was determined in an adult reference Caucasian population (n=70) composed of 26 females and 44 males aged 19–78 years (mean 43.6). Results ranged from 7.8 to 27.8 nmol h^{-1} ml^{-1} packed red blood cells (PRBC) with a mean of 18.2. The results are similar to those previously published [1,4,6,7] (Table 2).

The frequency histogram (Fig. 3) shows a bimodal distribution with about 10% of intermediate TPMT activity. This assay permits to measure TPMT activity in patients treated with 6-mercaptopurine or azathioprine and to determine the effect of genetic polymorphism on the treatment efficacy.

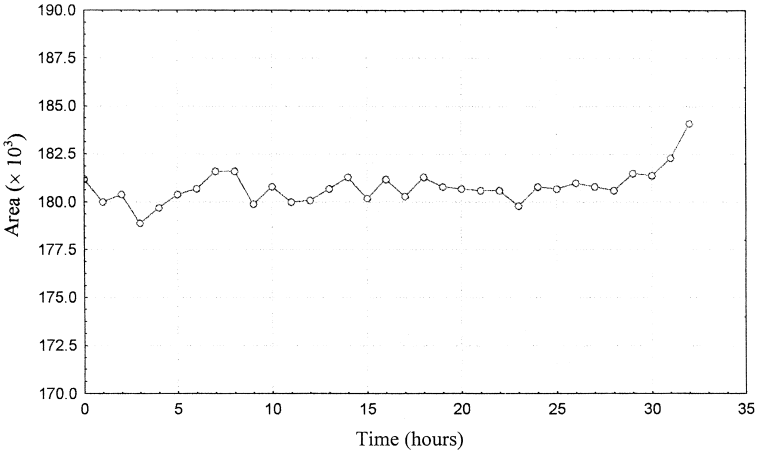

Fig. 2. Stability of the samples after extraction at room temperature; some samples are pooled just before chromatography and an aliquot is analysed every hour: area of the 6-MMP peak versus time (h).

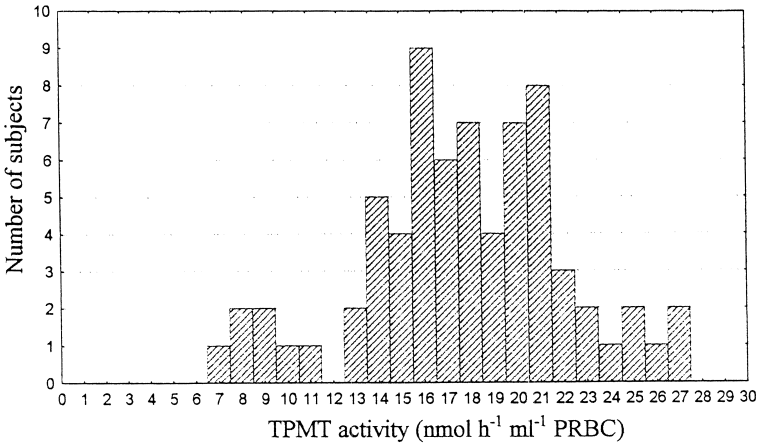

Fig. 3. Frequency distribution histogram of TPMT activity in an adult reference Caucasian population (n=70).

References

[1] R.M. Weinshilboum, S.L. Sladek, Am. J. Hum. Genet. 32 (1980) 651–662.
[2] L. Lennard, J.S. Lilleyman, J. Van Loon, R.M. Weinshilboum, Lancet 336 (1990) 225–229.
[3] R.M. Weinshilboum, F.A. Raymond, P.A. Pazmino, Clin. Chim. Acta 85 (1978) 323–333.
[4] E. Jacqz-Aigrain, E. Bessa, Y. Medard, Y. Mircheva, E. Vilmer, Br. J. Clin. Pharmacol. 38 (1994) 1–8.
[5] Y. Medard, S. Nafa, E. Jacq-Aigrain, J. Chromatogr. B 700 (1997) 275–277.
[6] B. Klemetsdal, E. Toffefsen, T. Loennechen, K. Johnsen, E. Utsi, K. Gisholt, E. Wist, J. Aarbakke, Clin. Pharmacol. Ther. 51 (1992) 24–31.
[7] M. Tinel, A. Berson, D. Pessayre, P. Letteron, M.P. Cattoni, Y. Horsmans, D. Larrey, Br. J. Clin. Pharmacol. 32 (1991) 729–734.