ELSEVIER

Journal of Chromatography B, 716 (1998) 392–396

JOURNAL OF CHROMATOGRAPHY B

Short communication

Reversed-phase high-performance liquid chromatographic assay method for quantitating 6-mercaptopurine and its methylated and non-methylated metabolites in a single sample

Hideo Mawatari*, Yasuhiro Kato, Shin-ichiro Nishimura, Nobuo Sakura, Kazuhiro Ueda

Department of Pediatrics, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

Received 23 June 1997; received in revised form 2 July 1998; accepted 2 July 1998

Abstract

Methods of assaying 6-mercaptopurine (6MP) and its methylated and non-methylated metabolites are essential for the therapeutic dose in treating patients with acute lymphoblastic leukemia. However, previous methods are technically complicated and unsuitable for clinical use. Thus, we have now developed a method utilizing reversed-phase high-performance liquid chromatography (HPLC) in order to quantify these compounds in human red blood cells (RBCs) in a single sample to serve as an index of cytotoxic activity. The agents 6MP, 6-thioguanine (6TG) and 6-methylmercaptopurine (6MMP) were well separated by this assay. Linear relationships were observed between the peak areas and the RBC concentrations of 6MP, 6TG and 6MMP over the range of 20–2000, 18–1800 and 18–1800 pmol per 25 mg hemoglobin (Hb), respectively. The limit of quantitation of the assay is 20, 18 and 18 pmol per 25 mg Hb, respectively. This assay system is suitable for routine clinical use.

Keywords: 6-Mercaptopurine; 6-Thioguanine; 6-Methylmercaptopurine

1. Introduction

6-Mercaptopurine (6MP) is widely used in the conventional chemotherapy of patients with acute lymphoblastic leukemia (ALL). Since few pharmacokinetic studies of 6MP have been performed, adjustment of the dose of 6MP is based primarily on evidence for myelosuppression. 6MP has a very short half-life in plasma and is rapidly eliminated. In contrast, its intracellular metabolites 6-thioguanine (6TG) and 6-methylmercaptopurine (6MMP) have long half-lives in red blood cells (RBCs) and they remain in the RBCs throughout their life span (Fig. 1). The RBC concentration of the active 6MP metabolite, 6TG, can therefore be used as an index of the cytotoxic activity of 6MP [1].

A major factor that influences the formation of 6TG is 6MP S-methylation, which is catalyzed by the enzyme thiopurine methyltransferase (TPMT). Humans exhibit wide individual genetic variations in the activity of TPMT in RBCs [2]. The activity of TPMT in human liver and normal lymphocytes has been shown to correlate with that in RBCs [3,4]. The methylated metabolites of 6MP, including 6MMP, are

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00329-6

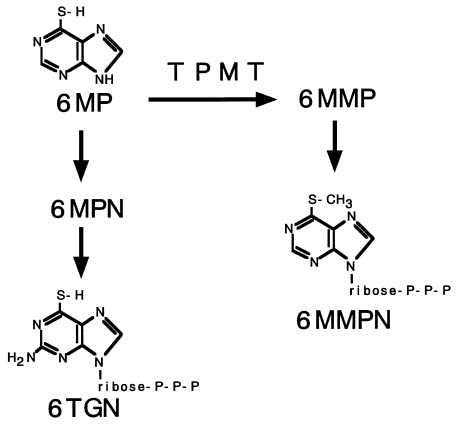

Fig. 1. Metabolites of 6-thiopurines. 6MP; 6-mercaptopurine, 6MPN; 6-mercaptopurine nucleotide, 6TG; 6-thioguanine nucleotide, 6MMP; 6-methylmercaptopurine, 6MMPN; 6-methylmercaptopurine nucleotide, TPMT; thiopurine methyltransferase

also cytotoxic [5], and it is the total amount of both methylated and non-methylated metabolites that is thought to be critical in the efficacy of 6MP.

Methods utilizing high-performance liquid chromatography (HPLC) have been developed for the determination of 6MP and its non-methylated metabolites [1,6–11]. However, other methods are required to assay the methylated metabolites of 6MP [12,13]. These systems are unsuitable for large prospective studies, due to their technical complexity and the number of different analytical set-ups required. A rapid and simple method for the analyses of 6MP and its methylated and non-methylated metabolites would, therefore, be extremely useful.

We developed a technique to assay 6MP, 6TG and 6MMP in a single sample, utilizing gradient method and changing the detection wavelength. Moreover, our method requires no solvent extraction. This assay is sensitive and suitable for routine clinical use.

2. Materials and methods

2.1. Subjects

Specimens of blood were drawn from a patient with ALL who was receiving 6MP (50 mg m^{-2} day^{-1}).

2.2. Reagents

6-Mercaptopurine (6MP), 6-thioguanine (6TG), 6-methylmercaptopurine (6MMP) and dithiothreitol (DTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA), and 1-heptanesulphonic acid sodium salt was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methanol (HPLC grade), water (HPLC grade) and perchloric acid (reagent grade) were obtained from Nakalai Tesque Inc. (Kyoto, Japan).

2.3. HPLC

Reversed-phase HPLC was performed using a composite system consisting of a pump (LC-10AD), a UV detector (SPD-10A), a column oven (CTO-10A) and a chart recorder (C-R7Ae plus), all from Shimadzu, Kyoto, Japan. This UV detector could change detection wavelength automatically. The column was an ODS-II (Shimadzu, Kyoto, Japan); particle size 5 μm, 150 mm in length, and 6.0 mm in diameter.

Analyses were performed at 40°C in 0.02 *M* phosphate buffer (pH 6.4), 0.1% 1-heptanesulphonic acid sodium salt. The concentration of methanol in the elution solvent was varied from 0% at 4 min to 14% at 15 min using a linear gradient profile. The total run time, including the equilibration time for the next run, was 35 min. The flow-rate was 2.5 ml min^{-1}, and the detection wavelength was changed from 332 nm (for 6MP and 6TG) to 289 nm (for 6MMP) at 12 min.

2.4. Preparation of samples

Blood samples (1.5 ml) were collected in heparinized tubes containing 1 mg DTT to protect the thiol group from oxidation. The samples were immediately centrifuged at 1000×*g* for 10 min at 4°C. Plasma, leukocytes and the upper layer of the erythrocytes were removed. The remaining erythrocytes were washed twice in saline and diluted with two times volumes of 0.02 *M* KH_2PO_4. A 1 ml aliquot was transferred to a tube containing 10 mg DTT, and the samples were rapidly deproteinized by the addition of 100 μl of 70% perchloric acid. The deproteinized samples were centrifuged at 2000×*g*

for 10 min at 4°C. Twenty μl of the supernatant were injected into the column for analysis of thiopurine base and nucleoside composition.

The remaining supernatant was heated for 45 min at 100°C to hydrolyze the thiopurine nucleotides and nucleosides to their free bases. The hydrolysate was cooled and centrifuged, and 20 μl of the supernatant were injected into the column.

For estimation of the 6MP, 6TG and 6MMP concentrations, hemoglobin concentration was measured in each aqueous hemolysate using a hemoglobin B-test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan), and the 6MP, 6TG, and 6MMP concentrations in each sample were normalized to 25 mg hemoglobin (Hb).

2.5. Statistical analysis

Linearity was calculated by linear regression analysis.

3. Results

Utilizing our HPLC-based assay, we found that 6MP, 6TG and 6MMP were well separated (Fig. 2). No interfering substances were present in the heated RBCs obtained from untreated normal subjects (Fig. 2A). In the chromatograms obtained from normal RBCs spiked with 1000 pmol of 6MP, 900 pmol of 6TG and 900 pmol of 6MMP per 25 mg Hb (Fig.

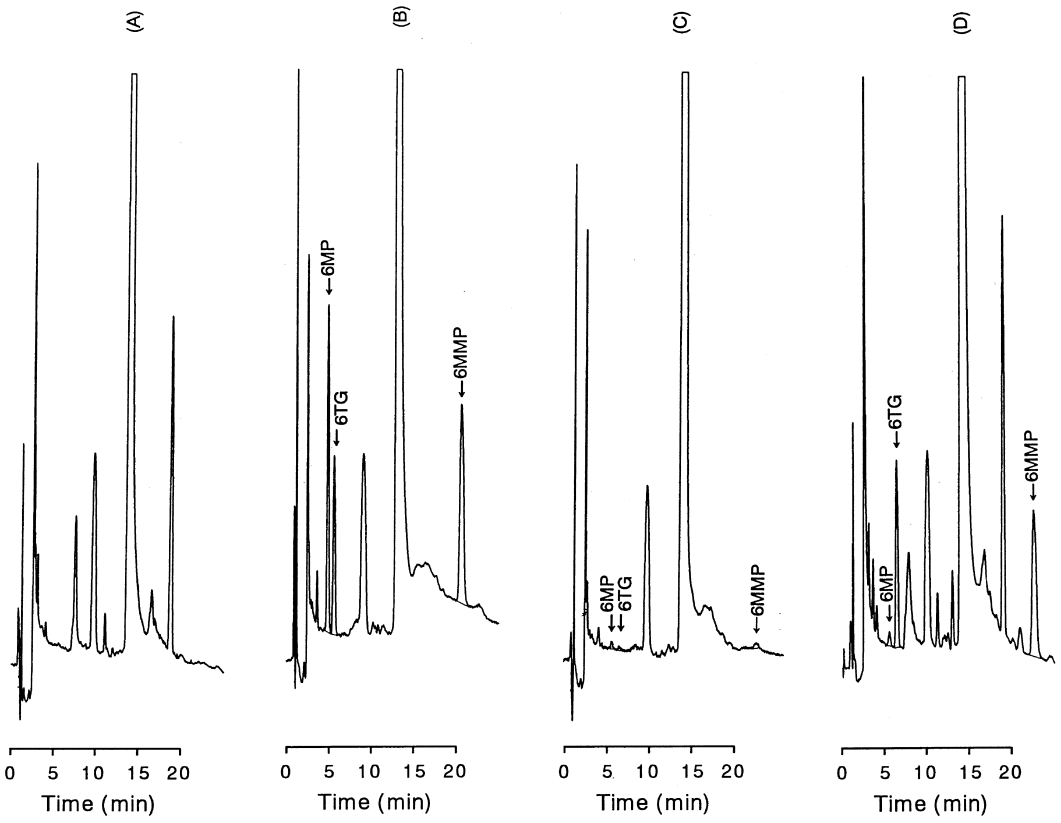

Fig. 2. Chromatograms of (A) heated normal RBCs, (B) normal RBCs spiked with 1000 pmol of 6MP, 900 pmol of 6TG and 900 pmol of 6MMP per 25 mg Hb eluted at 4.8, 5.5 and 20.9 min, respectively, (C) normal RBCs spiked with 20 pmol of 6MP, 18 pmol of 6TG and 18 pmol of 6MMP per 25 mg Hb (the lower detection limit) and (D) heated RBCs from a patient with ALL exhibited 41 pmol of 6MP, 707 pmol of 6TG and 605 pmol of 6MMP per 25 mg Hb.

2B), these three compounds eluted at 4.8, 5.5 and 20.9 min, respectively. The lower limit of detection for the assay was 20 pmol for 6MP, 18 pmol for 6TG and 18 pmol for 6MMP per 25 mg Hb (Fig. 2C). Heated RBCs from a patient with ALL who was being treated with 6MP exhibited 41 pmol of 6MP, 707 pmol of 6TG and 605 pmol of 6MMP per 25 mg Hb (Fig. 2D).

Linear relationships were observed between the peak areas and the concentrations of 6MP, 6TG and 6MMP over the range of 20–200 pmol, 18–1800 pmol and 18–1800 pmol per 25 mg Hb in the RBCs, respectively. The average equation obtained from standard curves was $y=6.416x-6.391$ ($r^2=0.999$, $P<0.001$) for 6MP, $y=5.025x-36.018$ ($r^2=0.999$, $P<0.001$) for 6TG and $y=10.738x+137.495$ ($r^2=1.000$, $P<0.001$) for 6MMP.

The reproducibility of the assay was evaluated from five measurements at each point of the calibration curve (intra-assay variation). The intra-assay coefficients of variation ranged from 0.3 to 9.1% (Table 1). When we assayed 1000 pmol of 6MP, 900 pmol of 6TG and 900 pmol of 6MMP per 25 mg Hb in RBCs four times over a 2-week period, we observed inter-assay coefficients of variation of 3.1%, 4.0%, 4.3%, respectively.

Although 6MMP was decomposed by heat, we observed a linear relationship between the concentration of heated (x) and non-heated (y) 6MMP; $y=8.949x-313.269$, $r^2=0.997$, $P=0.016$.

4. Discussion

Since 6MP is itself inactive, attention has been directed towards quantifying its active metabolite, 6TG, in RBCs. This is of considerable importance, since a low concentration of 6TG in the RBCs is considered to be a risk factor for relapse in children with ALL who are receiving 6MP as maintenance chemotherapy [1,14,15]. Ion-exchange chromatography has been used to separate the thionucleoside mono-, di-, and triphosphates [8]. The thionucleoside monophosphates of 6MP and 6TG have also been separated by ion-pairing chromatography [9]. More recently, thionucleotides have been analyzed after their transformation to thiopurine bases by acid hydrolysis, followed by a laborious double-step solvent extraction and reversed-phase HPLC [10,11]. Concentrations of 6MP, 6TG, 6MMP and 6-methylthioguanine were determined using two HPLC systems and two samples [12], and concentrations of 6MP, 6TG and 6MMP were assayed using two HPLC systems in a single sample [13].

Table 1
Intra-assay reproducibility in red blood cells

Compound	Concentration (pmol per 25 mg Hb)	Mean peak area	S.D.	C.V. (%)
6MP	20	120.4	8.39	7.0
	100	580.4	33.14	5.7
	200	1212.8	25.10	2.1
	1000	6632.2	54.00	0.8
	2000	12723.8	35.95	0.3
6TG	18	81.2	5.81	7.1
	90	359.2	30.03	8.4
	180	775.4	24.45	3.2
	900	4709.0	58.66	1.2
	1800	8909.0	57.99	0.7
6MMP	18	289.8	26.45	9.1
	90	1024.8	84.75	8.3
	180	2150.8	60.57	2.8
	900	9888.4	95.38	1.0
	1800	19419.0	123.85	1.4

The method described by Boulieu [11] was sensitive: 0.2 ng (1.3 pmol) for 6MP and 0.3 ng (1.8 pmol) for 6TG per 8×10^8 RBCs. The detection limit of the method described by Erdmann [12] was 30 ng (200 pmol) for 6MP, 5 ng (30 pmol) for 6TG, 50 ng (120 pmol) for 6MMP per 8×10^8 RBCs, and that by Lennard [13] was 30 pmol for 6MP, 30 pmol for 6TG, 120 pmol for 6MMP per 8×10^8 RBCs. The lower limit of detection for our assay method was 20 pmol for 6MP, 18 pmol for 6TG and 18 pmol for 6MMP per 25 mg Hb. Erythrocytes which were washed and centrifuged at $1000\times g$ for 10 min contained 8×10^8 cells per 100 μl. This number of cells corresponded to approximately 25 mg of hemoglobin. Thus, our method is as sensitive as previously described methods except the method by Boulieu, which is very laborious.

Peak area of heated 6MMP was smaller than that of non-heated 6MMP. It suggested that 6MMP was decomposed by heat. Since there was a linear relationship between the concentration of heated and non-heated 6MMP, it is possible to estimate 6MMP concentration in RBCs.

We have developed a single HPLC system of 6MP, 6TG, and 6MMP concentrations in a single sample, using gradient elution and changing the detection wavelength. This method did not require solvent extraction. The technical simplicity of this assay, and the elution of all chromatographic peaks within 25 min, make it highly useful in the study of large groups of patients, as well as for clinical use in evaluating individual patients.

References

[1] L. Lennard, J.S. Lilleyman, J. Clin. Oncol. 7 (1989) 1816.
[2] R.M. Weinshilboum, S.L. Sladek, Am. J. Hum. Genet. 32 (1980) 651.
[3] C.L. Szumlanski, R. Honchel, M.C. Scott, R.M. Weinshilboum, Pharmacogenetics 2 (1992) 148.
[4] J.A. Van Loon, R.M. Weinshilboum, Biochem. Genet. 20 (1982) 637.
[5] B.S. Tay, R.McC. Lilley, A.W. Murray, M.R. Atkinson, Biochem. Pharmacol. 18 (1969) 936.
[6] N.K. Burton, G.W. Aherne, V. Marks, J. Chromatogr. 309 (1984) 409.
[7] L. Lennard, J. Chromatogr. 345 (1985) 441.
[8] L.E. Lavi, J.S. Holcenberg, Anal. Biochem. 144 (1985) 514.
[9] S. Zimm, J.M. Strong, Anal. Biochem. 160 (1987) 1.
[10] L. Lennard, J. Chromatogr. 423 (1987) 169.
[11] R. Boulieu, A. Lenoir, J. Chromatogr. 615 (1993) 352.
[12] G.R. Erdmann, L.A. France, B.C. Bostrom, D.M. Canafax, Biomed. Chromatogr. 4 (1990) 47.
[13] L. Lennard, H.J. Singleton, J. Chromatogr. 583 (1992) 83.
[14] J.S. Lilleyman, L. Lennard, Lancet 343 (1994) 1188.
[15] K. Schmiegelow, H. Schrøder, G. Gustafsson, J. Kristinsson, A. Glomstein, T. Salmi, L. Wranne, J. Clin. Oncol. 13 (1995) 345.