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Short communication

Thiopurine methyltransferase activity: new high-performance liquid chromatographic assay conditions

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Abstract

This paper reports changes to our previously published high-performance liquid chromatographic method for the measurement of 6-methylmercaptopurine (6-MMP) in red blood cell lysates. The extraction procedure and chromatographic conditions have been improved and the range of the calibration curves has been modified. The recoveries of 10 and 100 ng ml^{-1} 6-MMP were 99.0±6.0% and 96.3±4.0% respectively and the limit of quantification was lowered to 5 ng ml^{-1}. This method, which does not require radioactive S-adenosyl-L-methionine, is more sensitive, specific and reproducible and may prove useful for routine determination of thiopurine methyltransferase activity in red blood cells. © 1997 Elsevier Science B.V.

Keywords: Enzymes; Thiopurine methyltransferase; 6-methylmercaptopurine

1. Introduction

Thiopurine drugs azathioprine, 6-mercaptopurine (6-MP) and 6-thioguanine are used to treat cancer patients and organ transplant recipients. Thiopurine methyltransferase (TPMT) catalysed S-methylation of 6-MP into 6-methylmercaptopurine (6-MMP) is a separate catabolic pathway, which involves S-adenosyl-L-methionine (SAM) as the methyl group donor [1]. TPMT activity is polymorphic and under genetic control, large inter-individual variations in 6-MP immunosuppressive activity and toxicity are inherited and involve differences in metabolism. Population studies have shown that TPMT activity is trimodally distributed, in a white Caucasian population 0.3% lack, 11% have intermediate and 89% have high TPMT activities [2]. Molecular cloning and structural characterization of the TMPT gene and elucidation of the molecular basis will help make it possible to develop DNA base diagnostic tests of a common polymorphism of this enzyme [3].

We have previously reported the measurement of the 6-MMP, product of the TPMT reaction, by a reversed-phase HPLC with UV detection [4]. This method was sensitive, specific and reproducible without the use of radioactive SAM and the results were correlated with those of the radiochemical reference method [5]. This paper reports a new

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extraction procedure with modifications in calibration range and HPLC conditions.

2. Methods

Materials, blood samples, preparation of erythrocytes, TPMT assay and incubation conditions are similar to those previously described by Jacqz-Aigrain [4].

Standard curves of 6-MMP (10–150 ng ml^{-1}) were prepared from appropriate dilutions of the stock solution of 6-MMP (1 mg ml^{-1}).

After the incubation phase, a new liquid–liquid extraction replaced the liquid–solid extraction. A 5-ml volume of dichloromethane–isopropyl alcohol (80:20, v/v) was added to each tube. The tubes were shaken gently for 15 min and centrifuged for 10 min at 3000 *g* (4°C). Then the aqueous layer was discarded and the organic phase was evaporated under nitrogen. The dry extract was dissolved in 250 μl of the mobile phase, mixed, filtered and centrifuged. An aliquot (50 μl) was injected into the HPLC system.

The analytical column was a Beckman ODS 2 C_{18}/5 μm (250×4.6 mm), the mobile phase of water–acetonitrile–triethylamine–DTT (92.7:7:0.2:-0.1, v/v) was adjusted to pH 3.2 with orthophosphoric acid and pumped at a flow-rate of 1.25 ml/min.

3. Results

The recoveries of 10 and 100 ng ml^{-1} 6-MMP added to red blood cell lysates were 99.0±6.0% (coefficient of variation (C.V.)=5.8%) and 96.3±4.0% (C.V.=4.6%) respectively (n=5).

The calibration range was modified (10–150 ng ml^{-1}) according to the 6-MMP concentrations detected after incubation. The 6-MMP calibration curves were linear with correlation coefficients >0.999 and a mean calibration graph (n=21) gave a regression of $y=5065.9x-4002.6$ (Fig. 1). The limit of quantification was reduced to 5 ng ml^{-1}, at a signal-to-noise ratio greater than 10. The inter-assay C.V. over the concentration range of 10–150 ng ml^{-1}, evaluated over 21 assays, ranged from 6.7–

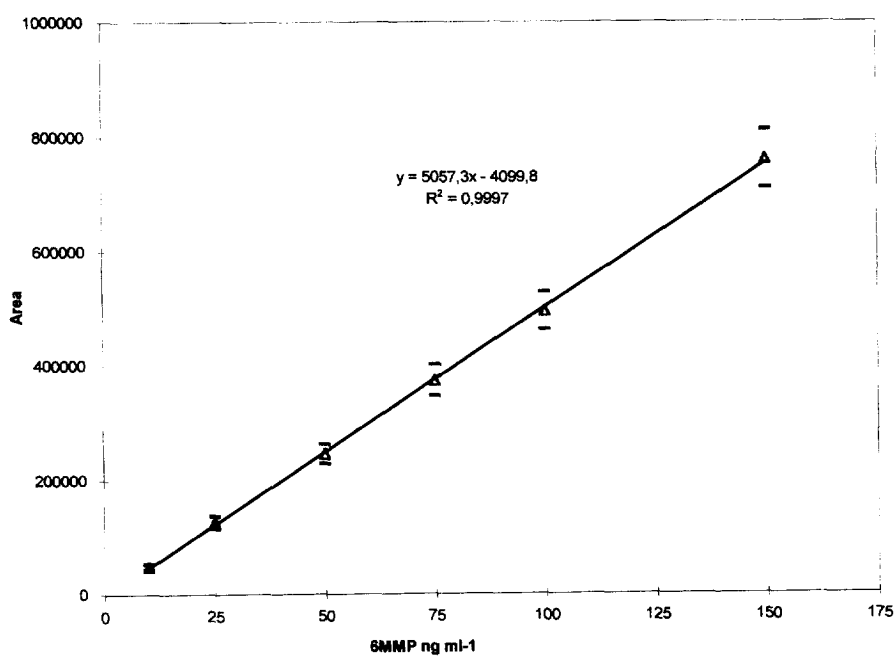

Fig. 1. Mean calibration curve (n=21) of 6-MMP concentrations (10–150 ng ml^{-1}) versus corresponding peak areas.

Table 1
Calibration curves of 6-MMP: inter-assay variation of 6-MMP concentrations

Calibration points	Concentration (ng ml^{-1}) $n=21$					
	10	25	50	75	100	150
Mean area	48 531	126 259	246 044	373 325	494 322	760 432
S.D.	4960	11 282	16 849	27 365	32 905	50 857
C.V. (%)	10.22	8.94	6.85	7.33	6.66	6.69

10.2%. Under the same conditions, the intra-assay C.V., evaluated over 5 assays, ranged from 2.7–6.9%. (Table 1).

A quality control lysate was included into each assay, intra-assay variation ($n=5$) was 2.6% and inter-assay variation ($n=5$) was 3.7%. Enzyme activity measured by the new HPLC assay ($n=7$ over one month) was 13.8 units TPMT, the activity of the same quality control by the previous HPLC assay was 14.5 units TPMT ($n=8$).

4. Discussion

TPMT activity was initially determined by the radiochemical reference method using radioactive SAM as the donor of methyl group [5]. We developed a HPLC assay following a liquid–solid extraction [4], also used by others [6] which was correlated with the radiochemical reference method. We later had to develop a new liquid–liquid extraction to improve recoveries of 6-MMP because liquid–solid extraction recovery fell under 50% with the new polymeric MP1 column modified by the manufacturer.

This new HPLC assay with liquid–liquid extraction had a better efficiency, sensitivity and was easier to perform at a lower cost. All these modifications improved an assay which is important for routine determination of TPMT activity, as this measurement may prove useful to optimise dosage regimens in patients with different TPMT activities and to prevent severe thiopurine toxicity in patients lacking TPMT activity.

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