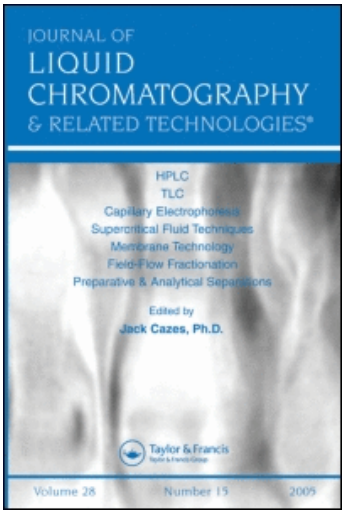


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Determination of Six Thioguanine Nucleotides in Human Red Blood Cells Using Solid-Phase Extraction Prior to High Performance Liquid Chromatography

J. L. Bonnefous[a]; Ph. Gendre[a]; M. Guillaumont[b]; A. Frederich[b]; G. Aulagner[a]
[a] Service pharmaceutique, Lyon Cedex, France [b] Laboratoire de pharmacologic, Hôpital DEBROUSSE, Lyon Cedex, France

**To cite this Article** Bonnefous, J. L. , Gendre, Ph. , Guillaumont, M. , Frederich, A. and Aulagner, G.(1992) 'Determination of Six Thioguanine Nucleotides in Human Red Blood Cells Using Solid-Phase Extraction Prior to High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 15: 5, 851 — 861
**To link to this Article: DOI:** 10.1080/10826079208018839
**URL:** http://dx.doi.org/10.1080/10826079208018839

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(5), 851-861 (1992)

# DETERMINATION OF SIX THIOGUANINE NUCLEOTIDES IN HUMAN RED BLOOD CELLS USING SOLID-PHASE EXTRACTION PRIOR TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J. L. BONNEFOUS[1], Ph. GENDRE[1], M. GUILLAUMONT[2], A. FREDERICH[2], AND G. AULAGNER[1]
[1]*Service pharmaceutique*
[2]*Laboratoire de pharmacologie*
*Hôpital DEBROUSSE, 29 rue Soeur Bouvier*
*69322 Lyon Cedex 05, France*



## ABSTRACT

A sensitive and rapid assay for the 6 thioguanine nucleotides (6TGN), major intracellular active metabolite of 6 Mercaptopurine, in human Red Blood Cells (RBCs) was developed. This assay is based on the hydrolysis of 6TGN to 6 Thioguanine (6TG) followed by a solid-phase extraction prior to HPLC analysis. A KROMASIL-C18 column was used to separate. Analytical recovery for 6TGN was 58 % (coefficient of variation 6 %). The limit of detection was 25 pmol per $8.10^8$ RBCs The assay was linear within the range 25-1600 pmol per $8.10^8$ RBCs. The advantage of this technique is that it does not require any toxic solvant. This assay may contribute to monitoring the patient's compliance in leukemic children undergoing 6 Mercaptopurine therapy.

## INTRODUCTION

6 Mercaptopurine (6MP) is used in the maintenance of remission in childhood acute lymphoblastic leukemia. 6MP is a prodrug which is activated to 6 thio-inosinic acid (6TIA) and metabolized to thioxanthine and thioguanine nucleotides. The cytotoxicity of 6 MP is related to the incorporation of 6TGN into DNA (1). 6 TGN is the major active metabolites in Red Blood Cells (RBCs) (2) and RBCs 6 TGN concentration is a better index of the antileukemic effect of 6MP than 6MP plasma concentration (3)



In order to determinate the RBCs levels of nucleotides with HPLC, two types of methods are now available. The first one consist of extracting and analyzing RBCs nucleotides. Several assays were reported using flow-fluorometric HPLC (4), anion-exchange chromatography (5) or ion-pair HPLC (6). These techniques involve multiple steps and methodological problems, they are time consuming and can't be used in routine clinical practice.

The second type of methods consists of hydrolysing nucleotides to parent thiopurine and then determining the 6 Thioguanine (6TG) total concentration. Two techniques were reported (7-8). These techniques involve a liquid-liquid extraction prior HPLC analysis. LENNARD's method (7) uses toxic solvants (Toluene, Phenyl Mercury Acetate) followed by acid back-extraction ; in the ERDMANN et al method (8) no toxic solvants are used during the extraction but this method requires fluorimetric detection.

This paper presents a rapid and selective method to determinate RBCs 6TGN using solid-phase exteaction after having hydrolysed 6TGN to 6TG and prior to an HPLC analysis.

## MATERIALS AND METHODS

### Reagents

6TG, 6MP, 6 Thioxanthine (6TX), 6 TIA and dithiothreitol (DTT) were obtained from Sigma Chemical Company (L'Isle d'Abeau, France). 6 Thioguanine Monophosphate (6TGMP) was generously given by Wellcome (Research Triangle Park, NC, USA). Methanol, Acetonitrile, Sulphuric acid, Phosphoric acid, Hydrochloric acid and Natrium acetate were purchased from MERCK (Nogent-sur-Marne, France). Sodium Hydroxide came from Prolabo (Paris, France).

Stock solutions (100 mg/l) were prepared by dissolving 10mg 6TG or 6TX in 4ml of sodium hydroxide (0,1M) and adding water up to 100 ml. For the 6MP, 6TIA or 6TGMP, before adding up water, 10 ml of hydrochloric acid (1M) will be poured in the solution.



### HPLC procedure

The HPLC system consisted of a SCL-6A system controller module equipped with a solvent delivery unit LC-6A, a sample injector SIL-9A, a detector SPD-6AV and a data processing units C-R 3A (all from SHIMADZU, Touzart et Matignon, Vitry sur Seine, France).

The analysis was performed on a KROMASIL-C18 column (250mm x 4.6mm ID, 5µm particle size) (Touzart et Matignon, Vitry sur Seine, France).

The mobile phase was water adjusted to pH 2.5 with phosphoric acid and contained a 1mM concentration of dithiothreitol.

The flow-rate was 1.ml/min and the detection wavelength was 342nm. The column was flushed daily with methanol-water (50:50, v/v).

## Sample collection and preparation

RBCs were obtained from heparinized whole blood which was centrifuged at 1500 tr/min for 10 min at 4°C. The erythrocytes were washed twice in a volume of NaCl 9‰ and centrifuged at 1500 tr/min for 10 min at 4°C. The RBCs were diluted in one volume of NaCl 9‰ and aliquots of 300µl were stored at -20°C. A dilution (1:10 in Isoton) was made for the count of RBCs.



## Extraction

The extraction involves two steps. The first step was the hydrolysis of 6TGN. To 200µl of RBCs, we added 500µl of 1,5M sulphuric acid, 500µl of water and 300µl of 10mM DTT in round-bottomed test-tube. The mixture was heated for 1h at 100°C. After cooling it down, 300µl of 5M sodium hydroxide were added and the tubes were stired up. The samples were centrifuged and the supernatant was filtered.

The second step is the solid-phase extraction of the thiopurine bases. The extraction system consisted of solid-phase extraction columns, Sep Pak C18 Classic Cartridges, 360 mg (Waters Millipore, Saint Quentin-Yvelines, France) and the Visiprep$^{R}$ solid-phase extraction,vacuum manifold (SUPELCO, Saint-Germain en Laye, France). The columns were activated with 3ml of methanol followed by 3ml of acetate buffer 35mM (pH : 4.75). The filtered supernatant (1ml) was passed through the columns. After air drying columns, elution was performed with 1.2ml of acetonitrile-water (80:20, v/v). The eluate was

evaporated to dryness under nitrogen at 40°C and the residue was reconstituted with 200µl of mobile phase to which 100µl were injected.

## Quantification

No suitable internal standard could be found for this assay, so we decided to add 6MP as internal standard in the calibration curve and each patient RBCs sample was duplicated adding the 6MP in one tube and nothing in another. Indeed in some patients, we had found a peak of 6MP.



# RESULTS AND DISCUSSION

## Chromatography

With our chromatographic system we can separate the three thiopurines (6TG, 6MP, 6TX) as shown in figure 1. The resolution between these compounds increased as the pH decreased. The KROMASIL C18 is obtained using monofunctional silane which gives high chemical stability (between pH 1.5 and 9.5) and allows the use of mobile phase at pH 2.5. Dithiothreitol (DTT) is a reducing agent which stabilizes the thiol groups. Without addition of DTT, the compounds were unstable in the mobile phase.

## Standard curve

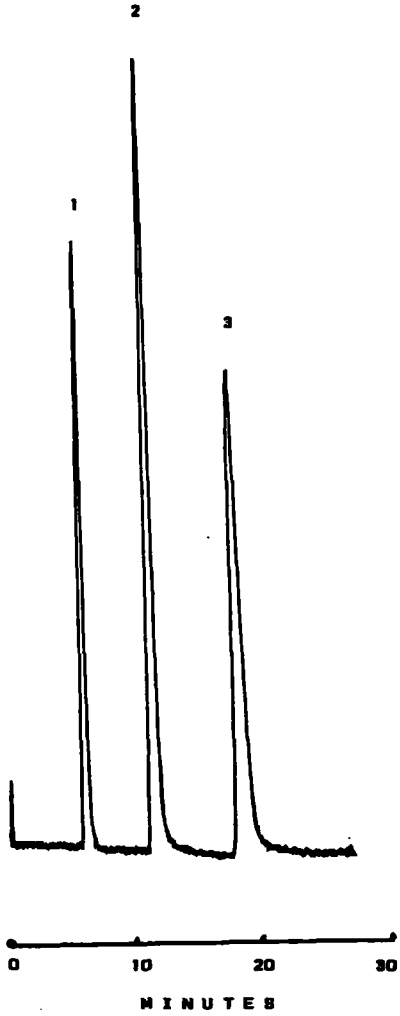


Figure 1. Chromatographic separation of mixture containing 6TG (peak 1), 6MP (2) and 6TX (3).

## TABLE I

### Precision and Recovery

| Compound | Concentration pMol per $8.10^8$ RBCs | Coefficient of variation CV (%) | | Recovery % | CV % |
|---|---|---|---|---|---|
| Intra assay (n = 5) | | | | | |
| 6TG | 100 | Height | 8.1 | 57.1 | 8.1 |
| | | Ratio Height | 5.6 | | |
| | 400 | Height | 5.9 | 58.6 | 5.9 |
| | | Ratio Height | 3.6 | | |
| 6MP | 1300 | Height | 4.8 | 62.2 | 4.8 |
| Inter assay (n = 5) | | | | | |
| 6TG | 100 | Height | 11.8 | 58.4 | 8.6 |
| | | Ratio Height | 6.1 | | |
| | 400 | Height | 9.8 | 59.4 | 7.8 |
| | | Ratio Height | 6.2 | | |
| 6MP | 1300 | Height | 10.2 | 63.1 | 7.9 |



variation, CV : 2.3%). Good linearity was observed for 6TG within the concentration ranges studied (25 - 1600 pMol). The detection limit at a signal-to-noise ration of 4 was 25 pMol per $8.10^8$ RBCs

## Recovery and precision

Recoveries were calculated by comparing the peak height of compounds obtained from stock solutions and standard RBCs

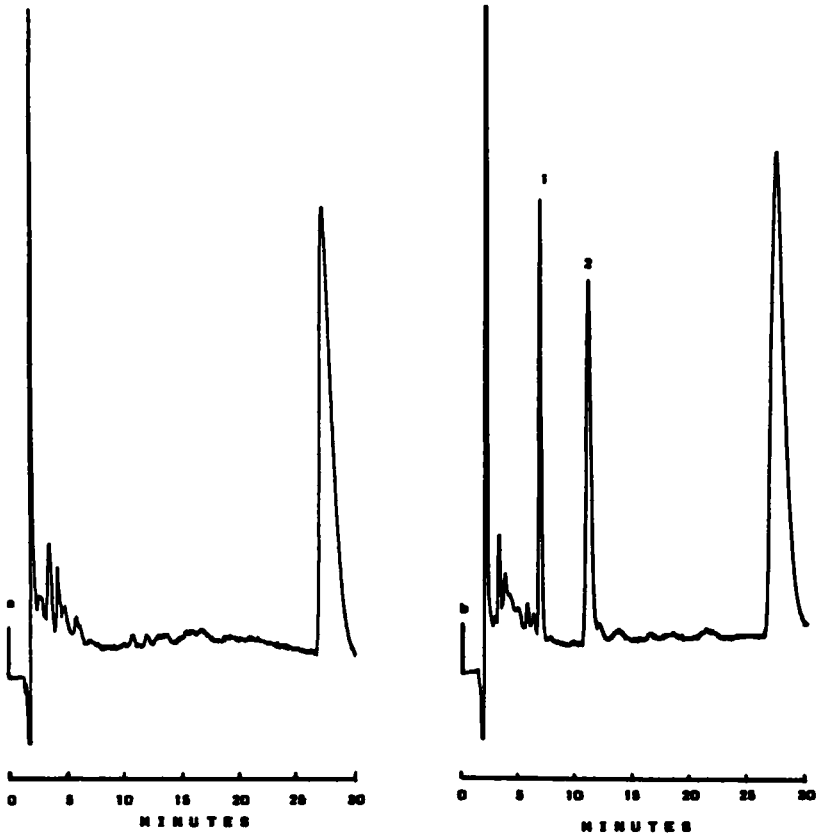


Figure 2. Chromatograms of blank RBCs (a) and RBCs standard (b) spiked with 6TG (peak 1) at 400 pMol. and 6MP (peak 2) at 1300 pMol.

samples after extraction. The intra-assay and inter-assay results are given in table I. The coefficients of variation are better when using 6MP as an internal standard.

Chromatograms of RBCs standard spiked with 6TG and 6MP and blank RBCs are shown in figure 2. An endogenous red blood cells compound was eluated at 28 min.

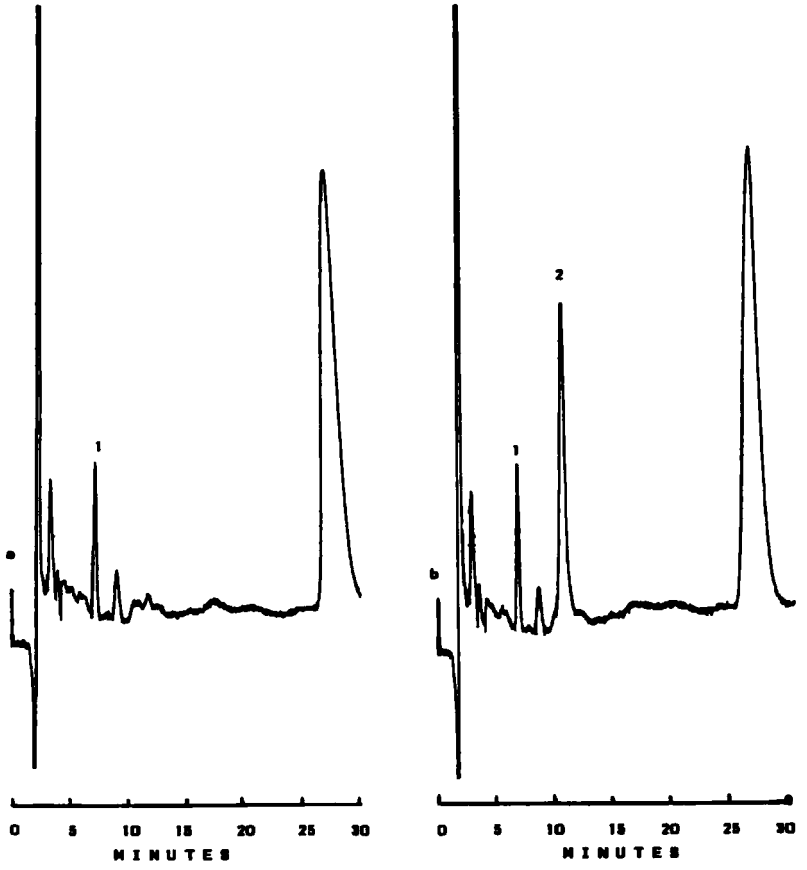


Figure 3. Chromatograms of RBCs sample from leukemic child undergoing 6MP therapy, without addition of 6MP (a) and with addition of 6MP (b).
Peaks 1: 6TG (155 pMol per $8.10^8$ RBCs); 2: 6MP.

No interference could be observed with methotrexate co-administered for the maintenance of remission in childhood acute lymphoblastic leukemia.

## Patient sample

This assay was used to determine RBCs 6TGN concentration in children receiving 6MP. Both chromatograms (without and with addition of 6MP) are shown in figure 3.

In conclusion, this technique -based on a solid-phase extraction, after an acid hydrolysis of 6TGN to 6TG and prior to HPLC analysis- is a suitable method for routine clinical practice in childhood acute lymphoblastic leukemia. It is a rapid assay and offers the advantage of using a no toxic solvant.



## ACKNOWLEDGEMENT

we thank Dr. R. CAPDEVILLE and Pr. N. PHILIPPE (DEBROUSSE hospital) for the provision of patient samples.

## REFERENCES

1 - TIDD D.M. and PATERSON A.R.P., Cancer Res., 34, 738, 1974.
2 - LENNARD L. and MADDOCKS J.L., J. Pharm. Pharmacol., 35, 15, 1983.
3 - LENNARD L, KEEN D. and LILLEYMAN J.S., Clin. Pharmacol. Ther., 40, 287, 1986.
4 - DOOLEY T. and MADDOCKS J.L., J. Chromatogr., 229, 121, 1982.

5 - LAVI L.E., and HOLCENBERG J.S., Anal. Biochem., 144, 514, 1985.
6 - ZIMM S. and STRONG J.M., Anal. Biochem., 160, 1, 1987.
7 - LENNARD L., J. Chromatogr., 423, 169, 1987.
8 - ERDMANN G.R., FRANCE L.A., BOSTROM B.C. and CANAFAY D.M, Biomed. Chromatogr., 4, 47, 1990.