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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF 6-METHYLTHIOGUANINE, A MAJOR METABOLITE OF 6-THIOGUANINE, IN URINE

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## SUMMARY

A flow-fluorimetric high-performance liquid chromatographic assay for 6-methylthioguanine in urine has been developed. This compound is a major catabolite of 6-thioguanine, an important drug in cancer chemotherapy. The metabolite was extracted from alkaline urine with ethyl acetate which was injected onto a reversed-phase high-performance liquid chromatographic system for separation and detection. The method is simple, rapid and sensitive to below 500 ng $ml^{-1}$ which is below the levels encountered following a therapeutic dose of 6-thioguanine. Another metabolite was chromatographically separated from 6-methylthioguanine and partially characterised.

## INTRODUCTION

Thioguanine (TG) is widely used in the treatment of acute myeloid leukaemia, yet there is little known of its metabolism in patients. After an oral dose of [$^{35}$S]TG up to 40% is excreted in urine in 24 h [1] largely as inorganic sulphate with 6-methylthioguanine (MTG) and 6-thiouric acid (TU). The structures of TG and some of its metabolites are given in Fig. 1. There is a large variation between patients in the fraction of the dose excreted, possibly due to metabolic differences [1, 2]. Moreover, there have been reports of quantitative differences in excreted metabolites according to the disease being treated. Patients with leukaemia excreted much more MTG than TU [1] whereas in those with solid tumours the reverse was true [3]. Further, malignant disease associated alterations in purine metabolism (ring methylation) have been reported [4, 5].

It is possible, therefore, that increased methylation of TG might be a useful

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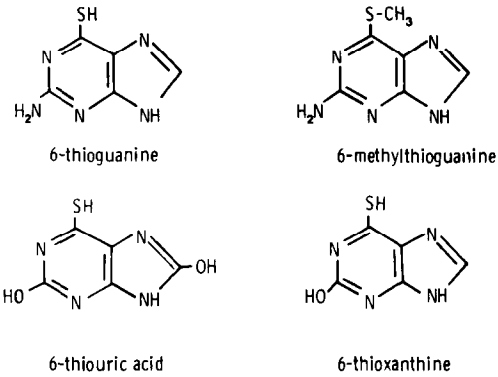


**Fig. 1. Structures of 6-thioguanine and some of its metabolites.**

marker for leukaemic cells. To investigate this we have developed a new assay for urinary MTG, suitable for clinical studies. The method involves solvent extraction and injection of the extract onto a reversed-phase high-performance liquid chromatographic (HPLC) system.

Another urinary metabolite of TG was separated from MTG and partially characterised through its resemblance to a product from the methylation of 6-thioxanthine (TX).

*Chromatography*

MTG was separated from urine constituents and other metabolites by extraction into ethyl acetate followed by HPLC using an ODS ($C_{18}$) reversed-phase on Spherisorb (particle size 5 $\mu$m) column, dimensions 250 mm × 4.8 mm, with a solvent system of 5% glacial acetic acid in methanol—water, pH 2.8 (60:40) at a flow-rate of 1 ml $min^{-1}$ using an Altex Model 110A pump.

The separated MTG was detected using a Shoeffel FS-970 flow-spectrophotofluorimeter, exciting at 330 nm and measuring emission through a 389-nm cut-off filter. No other filters were used. The photomultiplier output was measured on a Servoscribe 1S flat-bed recorder. The sample injector was a Waters Model U6K. All liquid volumes were delivered using Gilson automatic pipettes P20 to P5000.

As standard practice in our laboratories, all glass tubes used in fluorimetric analyses were decontaminated in 30% nitric acid for over 15 h and rinsed in glass-distilled water.

*Chemicals and reagents*

6-Thiouric acid (TU) was obtained from Calbiochem (Hertfordshire, U.K.). 6-Thioguanine (TG), 6-mercaptopurine (6MP), 6-methylmercaptopurine (6MMP), 6-methylthioguanine (MTG) and 6-thioxanthine (TX) were obtained from Sigma (London, U.K.).

Methanol and ethyl acetate were obtained from Rathburn Chemicals (Peebleshire, U.K.). Trimethylanilinium hydroxide (MethElute, Pierce and Warriner, Cheshire, U.K.) was used as supplied (0.2 *M* in methanol). All water used in solution preparation was glass-distilled and deionised; all other reagents were standard analytical grade. Thin-layer chromatography (TLC) was carried out using Eastman-Kodak 13255 cellulose plates (without fluorescent indicator) and 13179 silica plates (without fluorescent indicator).

*Determination of urinary MTG concentrations*

Standard solutions of MTG in urine were prepared in the range 0.5—10 μg $ml^{-1}$ by the addition of a fixed volume (300 μl) of aqueous standard in the range 5—100 μg $ml^{-1}$ to urine (2.7 ml). These analytical standards were alkalinated by the addition of 1 *M* sodium hydroxide (500 μl) to each sample, and extracted with ethyl acetate (3 ml) by shaking on a linear agitator at 280 rpm for 10 min. After centrifugation at 800 *g* for 2—3 min the ethyl acetate layer was analysed by injection of an aliquot (100 μl) into the HPLC system described above (see *Chromatography*). MTG showed a retention time of approximately 8 min.

*Methylation of TG metabolites*

A sample of each of TU, TX TG, 6MP, and 6MMP (3—4 mg each, approximately 20 μmol) was dissolved in methanol (5 ml). Trimethylanilinium hydroxide (MethElute), 0.2 *M* in methanol (100 μl), was added and the mixture heated at 35°C for 40 min. Excess MethElute was destroyed by the addition of water (5 ml). For the methylation of TX in later experiments, the reactants were heated at 50°C for 1 h after a method described for the methylation of xanthine [6].

## RESULTS

A linear relationship between peak height and urine MTG concentration was seen. The mean inter-assay variation over all points was 3.8% with intra-assay variation for a single point at 3.0%.

As observed with guanine [7] the fluorescence of MTG in methanol—water mixtures was greater than in aqueous media. It was thought that the addition of methanol to urine might impart sufficient fluorescence to any MTG present

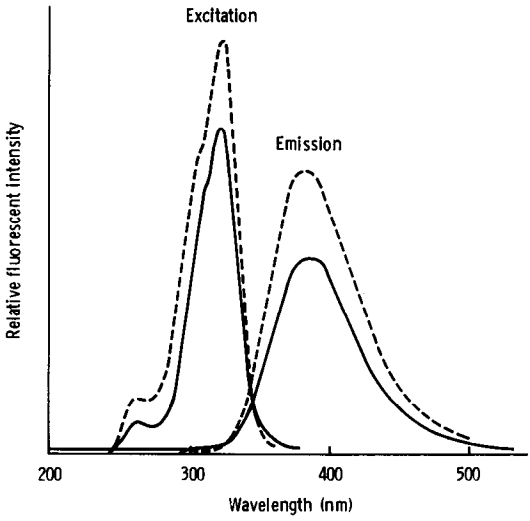


Fig. 2. Excitation and emission maxima of 6-methylthioguanine and a metabolite extracted from the urine of a patient following an oral dose of 6-thioguanine (160 mg). The spectra designated by a broken line correspond to 6-methylthioguanine.

to permit its fluorimetric detection. However, the urine constituents also showed quite intense fluorescence on excitation following the addition of methanol which prevented the detection of MTG. On extraction with ethyl acetate, MTG was found to be fluorescent in this solvent also and could be assayed by direct fluorimetry of the extract. Extraction was most efficient from alkaline urine (83%) and no detectable interference was encountered from TU and TG.

On extracting the urine from a patient receiving orally TG, however, an interfering metabolite was observed with similar fluorescent characteristics to MTG (Fig. 2). This was uncovered by cellulose thin-layer chromatographic (TLC) analysis with low-temperature luminescent detection [8]. MTG gave a higher $R_F$ when applied in dilute sodium hydroxide over that when applied in dilute hydrochloric acid, ethyl acetate or methanol, water being the eluent in each case (Table I). No such variation was observed with the urinary metabolite.

TABLE I

CELLULOSE TLC OF 6-METHYLTHIOGUANINE (MTG) AND A URINARY CATABOLITE OF 6-THIOGUANINE

All chromatograms were developed in water.

| Application medium | $R_F$ MTG | $R_F$ Metabolite |
|---|---|---|
| 0.1 *M* Hydrochloric acid | 0.16 | — |
| 0.1 *M* Sodium hydroxide | 0.38 | — |
| Ethyl acetate | 0.16 | 0.44 |
| 0.1 *M* Hydrochloric acid in urine | 0.20 | 0.44 |
| 0.1 *M* Sodium hydroxide in urine | 0.38 | 0.44 |

Using the HPLC solvent system methanol—water (60:40) MTG was separated from urine constituents following ethyl acetate extraction; however, the metabolite gave a very similar retention time with this solvent system (Fig. 3). In order to separate the metabolite from MTG, the pH of the solvent system was reduced by the addition of glacial acetic acid (5%). This increased the retention of MTG to approximately 18 min leaving the metabolite retention unchanged (Fig. 4). Increasing the flow-rate gave a retention time of 8 min for MTG (Fig. 5) and this was the system finally used for the assay of MTG.

*Identification of the urinary metabolite*

A number of thiopurines were methylated separately and the products from each reaction were analysed separately by HPLC. The chromatograms are described in Table II. The retention times for the parent thiopurines were also obtained individually with no attempt made to separate them chromatographically. A product from the methylation of TX had a similar retention time to that of the urinary metabolite in the mobile phase methanol—water (60:40), with and without the addition of 5% glacial acetic acid. A 6MP methylation product also had a similar retention time but unlike the metabolite and methylated TX this could not be detected fluorimetrically.

Although methylation might be expected to derivatise a number of other

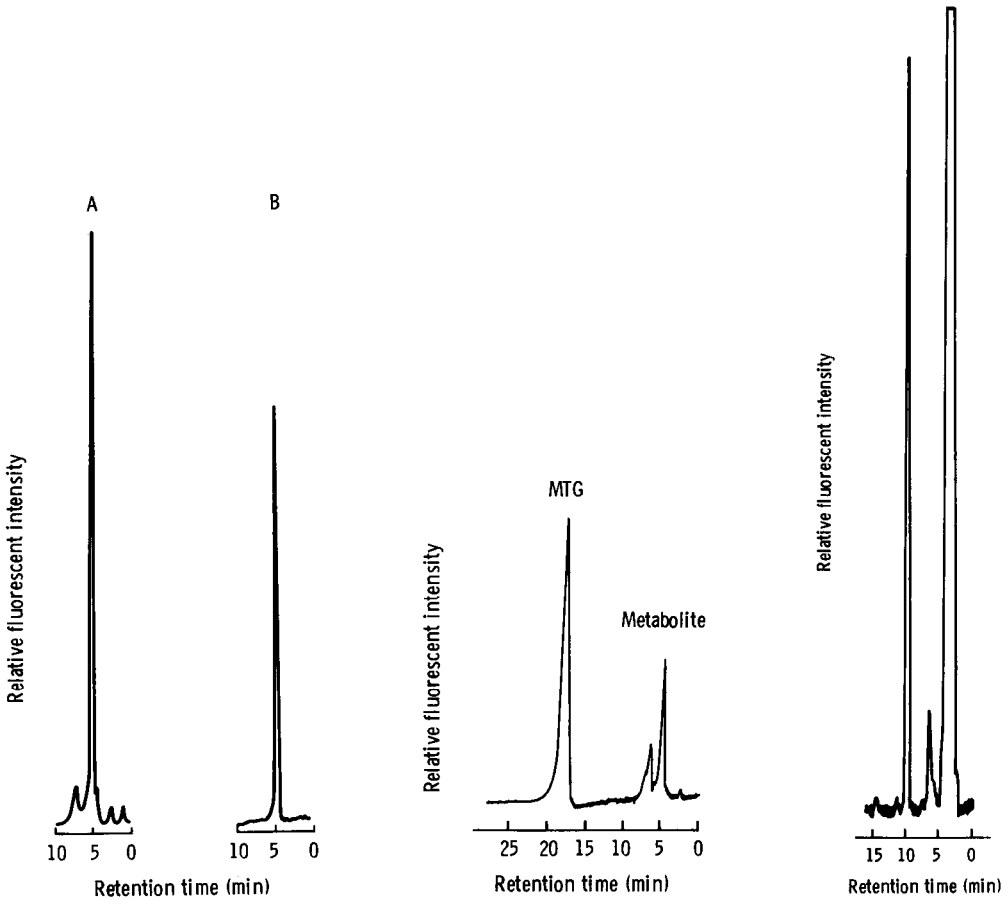


Fig. 3. Chromatograms of (A) 6-methylthioguanine extracted from urine, and (B) an extract from the urine of a patient treated orally with 6-thioguanine (160 mg). Chromatographic conditions: Whatman Sperhisorb 5 ODS (particle size 5 $\mu$m) reversed-phase ($C_{18}$) column (250 × 4.8 mm) with a methanol—water (60:40,v/v) mobile phase at a flow-rate of 0.4 ml $min^{-1}$.

Fig. 4. A chromatogram of 6-methylthioguanine (MTG) standard and a metabolite extracted from a patient's urine following an oral dose of 6-thioguanine (100 mg). Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 $\mu$m) reversed-phase ($C_{18}$) column (250 × 4.8 mm) with a solvent system of methanol—water (60:40, v/v) containing 5% glacial acetic acid at a flow-rate of 0.4 ml $min^{-1}$.

Fig. 5. A typical chromatogram of an extract from urine containing 6-methylthioguanine. Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 $\mu$m) reversed-phase ($C_{18}$) column (250 × 4.8 mm) with a solvent system of methanol—water (60:40, v/v) containing 5% glacial acetic acid at a flow-rate of 1 ml $min^{-1}$.

groups in the molecule [9] the fluorescent characteristics of these species are likely to differ from those of the S-methyl derivative [10]; hence any S-methyl TX may be selectively detected fluorimetrically in the presence of other methylation products. Further, on methylating TG in this way, a product showing similar fluorimetric and chromatographic properties to MTG was observed.

TABLE II

RETENTION TIMES OF SOME THIOPURINES AND THEIR METHYLATION PRODUCTS

Methylation conditions are described in *Methylation of TG metabolites*. Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 μm) reversed-phase ($C_{18}$) column (250 × 4.8 mm) with a solvent system methanol—water (3:2, v/v) at a flow-rate of 0.4 ml $min^{-1}$. Column effluent was analysed for UV absorbance using a Pye-Unicam LC 3 UV detector.

| Parent compound | Retention time (min) | Retention times (min) of methylation products |
|---|---|---|
| 6-Thioguanine | 4.0 | 4.2 |
| 6-Methylthioguanine | 4.2 | — |
| 6-Thiouric acid | 2.0 | 2.0 + late, broad peaks |
| 6-Thioxanthine | 2.5 | 3.8 |
| 6-Mercaptopurine | 2.5 | 3.8 + late, broad peaks |
| 6-Methylmercaptopurine | 3.0 | 4.3 + late, broad peaks |

## DISCUSSION

This assay for MTG is suitable for both routine clinical analysis and metabolic studies. To the knowledge of the authors, no such method has been described to date.

The potential pharmacokinetic importance of MTG urine concentrations has been indicated by its occurrence as a major catabolite of TG together with evidence showing that it may be the precursor for inorganic sulphate formation, the overall major urinary metabolite of TG [11, 12].

Following induction chemotherapy for leukaemia, the alteration in TG metabolism resulting in decreased urinary MTG concentrations could provide a marker for the achievement of remission thus avoiding unnecessary prolongation of the use of cytotoxic drugs. After remission, a state of relapse can only be ascertained clinically through the current methods of blood and bone marrow evaluation which require that the disease be relatively advanced [13]. This delay in diagnosis could be avoided if the increased methylation of TG associated with malignancy proves to be a sensitive indicator of the early stages of proliferation of the disease.

A body of information is emerging, pointing to a number of specific changes in purine and nucleic acid metabolism which occur at the onset of neoplastic disease [4, 5, 14]. Assay systems will be required if these metabolic changes are to be monitored and observations so obtained may provide powerful diagnostic tools for the management of malignant disease.

## ACKNOWLEDGEMENT

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## REFERENCES

1 G.A. LePage and J.P. Whitecar, Jr., Cancer Res., 31 (1971) 1627.

2 G.B. Elion, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 26 (1967) 898.
3 E.R. Lefkowitz, W.A. Creasey, P. Calabresi and A.R. Sartorelli, Cancer Res., 25 (1965) 1207.
4 R.W. Park, J.F. Holland and A. Jenkins, Cancer Res., 22 (1962) 469.
5 E. Borek, O.K. Sarma and J.I. Brewer, Amer. J. Obstet. Gynecol., 146 (1983) 906.
6 E. Brochmann-Hanssen and T. Olawuyi Oke, J. Pharm. Sci., 58 (1969) 370.
7 H.C. Borressen, Acta Chem. Scand., 19 (1965) 2100.
8 J.L. Maddocks and G.S. Davidson, Brit. J. Clin. Pharmacol., 2 (1975) 359.
9 J.L. Day, L. Tterlikkis, P. Grenier and L.J. Harold, J. Chromatogr., 177 (1979) 118.
10 H.C. Borressen, Acta Chem. Scand., 17 (1963) 2359.
11 G.B. Elion, S.W. Callahan and G.H. Hitchings, Proc. Amer. Assoc. Cancer Res., 3 (1962) 316.
12 G.B. Elion, R.W. Rundles and G.H. Hitchings, Proc. Amer. Assoc. Cancer Res., 5 (1964) 17.
13 W.N. Hittleman, L.C. Broussard, G. Dosik and K.B. McCredie, N. Engl. J. Med., 303 (1980) 479.
14 D.A. Heldman, M.R. Grever and R.W. Tewyn, Blood, 61 (1983) 291.