Journal of Chromatography, 229 (1982) 121—127
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1174

ASSAY OF AN ACTIVE METABOLITE OF 6-THIOGUANINE, 6-THIOGUANOSINE 5'-MONOPHOSPHATE, IN HUMAN RED BLOOD CELLS

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(First received September 14th, 1981; revised manuscript received November 19th, 1981)

SUMMARY

A flow-fluorimetric, liquid chromatographic assay for 6-thioguanosine 5'-monophosphate (TGMP) in human red blood cells (RBCs) has been developed. This compound is an active metabolite of 6-thioguanine, 6-mercaptopurine and the immunosuppressant, azathioprine.

The samples were prepared for chromatography by a novel isolation method which extracts the RBC constituents into an organic solvent and leaves the TGMP in the aqueous layer where it is oxidised to give a highly fluorescent species which is then separated by liquid chromatography.

The method is sensitive to below 200 ng ml^{-1} in RBCs which is below the levels encountered following a therapeutic dose of 6-thioguanine or azathioprine. The assay is simple and rapid enough for routine use.

INTRODUCTION

The initial step in the anabolism of the antileukaemic drug, 6-thioguanine is its conversion to 6-thioguanosine 5'-monophosphate (TGMP), which has been identified as the major metabolite accumulating in susceptible tumour and leukaemic cells [1]. This accumulation in leukaemic cells is probably important as most of the evidence indicates that 6-thioguanine exerts its cytotoxicity through the incorporation of TGMP into the cell DNA [2—4]. The mechanism of action of 6-mercaptopurine, another commonly used antileukaemic agent has also been attributed to its metabolism, via 6-thioinosine 5'-monophosphate, to TGMP, and its incorporation as such into DNA [5].

As the intracellular level of TGMP appears to be important for cytotoxic effects of both 6-thioguanine and 6-mercaptopurine, its measurement would be

0378-4347/82/0000—0000/$02.75

expected to yield clinically useful data concerning such factors as dosage schedules and resistance aquisition. In the absence of analytical techniques for such monitoring, valuable time may be lost by ineffective treatment through inadequate dosage or failure to identify a resistant leukaemia.

In an attempt to remedy this situation a simple, rapid assay for clinical RBC levels of TGMP has been developed using single solvent, anion-exchange liquid chromatography with flow-fluorimetric detection of the oxidised metabolite, following partial isolation from the RBC constituents by a novel solvent extraction method.

EXPERIMENTAL

Chromatography

Oxidised TGMP was separated from RBC constituents and other metabolites by anion-exchange chromatography using a Whatman Partisil 10 SAX (particle size 10 μm) strong anion-exchange column 250 mm × 4.8 mm I.D. (Jones Chromatography, Glamorgan, Great Britain), and an isocratic solvent system of methanol and 125 m*M* potassium phosphate—250 m*M* potassium chloride (1:2, v/v) at a constant flow-rate of 3 ml min^{-1} and a pressure of 200 bar using an Altex Model 110 A pump.

The separated TGMP was detected using a Schoeffel 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 at 5 mV full scale deflection. The sample injector was a Waters Model U6K. All liquid volumes were delivered using Gilson automatic pipettes P20 to P5000.

Glass tubes used in the extraction procedures were soaked for over 15 h in 30% nitric acid and rinsed in glass-distilled water.

Chemicals and reagents

6-Thioguanosine 5'-monophosphate was a gift from Dr. R.L. Miller of the Wellcome Research Laboratories (Research Triangle Park, NC, U.S.A.). Methanol and dichloromethane were obtained from Rathburn Chemicals (Peebleshire, Great Britain). Phenyl mercury acetate (laboratory agent) was obtained from BDH (Poole, Great Britain) and a 0.3% (w/v) solution in glass distilled, deionised water was prepared weekly. Tetrabutylammonium hydroxide (AnalaR, BDH) in a 40% aqueous solution and hydrogen peroxide (AnalaR, BDH) in a 6% aqueous solution were both used as supplied. A 0.24% w/v solution of potassium permanganate (AnalaR, BDH) in glass-distilled, deionised water was prepared as required. All water used in solution preparation was glass distilled and deionised; all other reagents used were standard analytical grade.

Determination of red blood cell TGMP concentrations

A standard solution of 10 μg ml^{-1} TGMP in water was prepared. Volumes of this (2—20 μl) were added to RBCs (100 μl) to give samples for analysis in the range 200—2000 ng ml^{-1}. This method of adding varying volumes of a fixed concentration standard obviously introduces a small dilution error (less than 1%).

To each of the above samples, 40% tetrabutylammonium hydroxide (100 μl), 5 *M* sodium hydroxide (20 μl) and 0.3% phenyl mercury acetate (200 μl) were added with vortex mixing after each addition. Dichloromethane (3 ml) was added and again each tube was mixed briefly. All tubes were then mixed in a multi-tube vortex mixer for 60 sec. The addition of tetrabutylammonium hydroxide before sodium hydroxide is recommended as this prevents the formation of large pockets of cellular protein which adheres to the tube on addition of dichloromethane leading to inconsistent extractions.

After extracting with dichloromethane, all tubes were then centrifuged at 1400 *g* for 10 min leaving the precipitated protein at the interface. A portion (200 μl) of the upper aqueous layer was then taken for analysis.

To each sample, 0.24% potassium permanganate (100 μl) was added and mixed. The TGMP was completely oxidised within 5 min; however, oxidation times in excess of 10 min led to degradation of the fluorophore. Excess permanganate was destroyed by addition of 6% hydrogen peroxide (10 μl) and the precipitated manganese dioxide thus formed was removed by centrifugation at 1400 *g* for 5 min. Aliquots from the supernatant were injected into the chromatographic system (see Chromatography) using 100 μl per injection.

Oxidised TGMP showed a retention time of approximately 3.5 min.

RESULTS AND DISCUSSION

The linear relationship between peak height and RBC concentration can be seen in Fig. 1. Reproducibility was assessed by measuring duplicate samples for each point in four separate experiments. The mean standard deviation for the whole group of measurements was 6.8% with the largest individual deviation at 7.6%.

Samples from patients receiving azathioprine following renal transplantation were analysed and a typical chromatogram is shown in Fig. 2. The large hump at a retention time of approximately 30 min in the patient's sample is believed, in the absence of standards, to be 6-thioguanosine diphosphate or an unresolved mixture of this and the triphosphate.

The oxidation of TGMP gives a highly fluorescent compound, probably the 6-sulphonate [6]. Detection of this compound in RBCs is not possible by direct fluorimetry due to interference from the physiological constituents. TGMP was separated from interfering substances by a novel isolation method followed by liquid chromatography of the oxidised compound. Using this system, levels of approximately 400 n*M* (200 ng ml^{-1}) are readily measurable which has been shown to be well below those encountered in clinical samples [7].

The extraction of TGMP into non-polar, organic solvents is not possible due to the presence of highly polar groups in the molecule. Phenyl mercury acetate has been shown to react with the thiol groups in 6-mercaptopurine [8] and 6-thioguanine [9] to give non-polar complexes which can be extracted into organic solvents. It was thought that by complexing TGMP in this way and forming an ion-pair with the phosphate group and tetrabutylammonium hydroxide, this might result in a non-polar species which could be extracted in

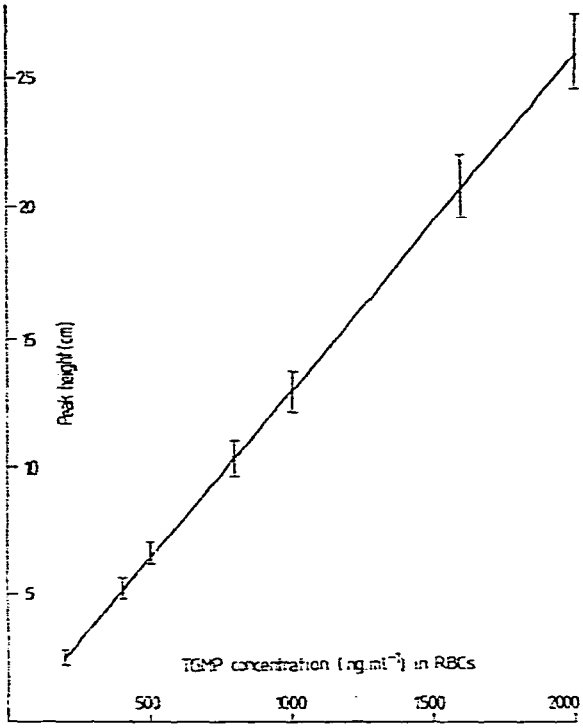

Fig. 1. Standard curve of concentration against peak height for oxidised TGMP from RBCs. Mean standard deviation for all readings was 6.8%, the largest individual deviation was 7.6%. Peak heights (± standard deviation) are converted by sensitivity factors to give readings as though they were in one instrumental sensitivity range.

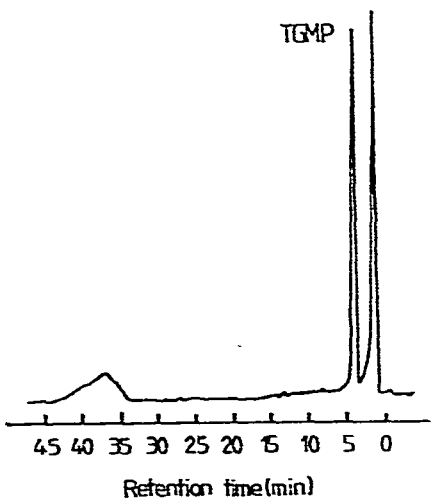

Fig. 2. Chromatogram of an extract from RBCs of a kidney transplant patient treated with azathioprine. The large hump at 30 min is believed to be the diphosphate or an unresolved mixture of the di- and triphosphate.

a similar way to 6-mercaptopurine and 6-thioguanine. However, the TGMP remained in the aqueous layer with no measurable amount in the organic phase which contained the bulk of the RBC constituents. This resulted in an excellent clean-up procedure, isolating TGMP from the RBC constituents for oxidation and chromatography.

Extraction using cold perchloric acid gave insufficient recovery (25%) from 10 μl of RBCs with 1 μg of TGMP.

Oxidation using acidic chromate was less efficient than alkaline permanganate in sample extracts.

Using slower flow-rates gave a longer retention time for TGMP with a better separation and improved sensitivity by approximately 50% (Fig. 3a—c) but it was felt that this increase in sensitivity was worth sacrificing to achieve a more rapid assay for clinical purposes.

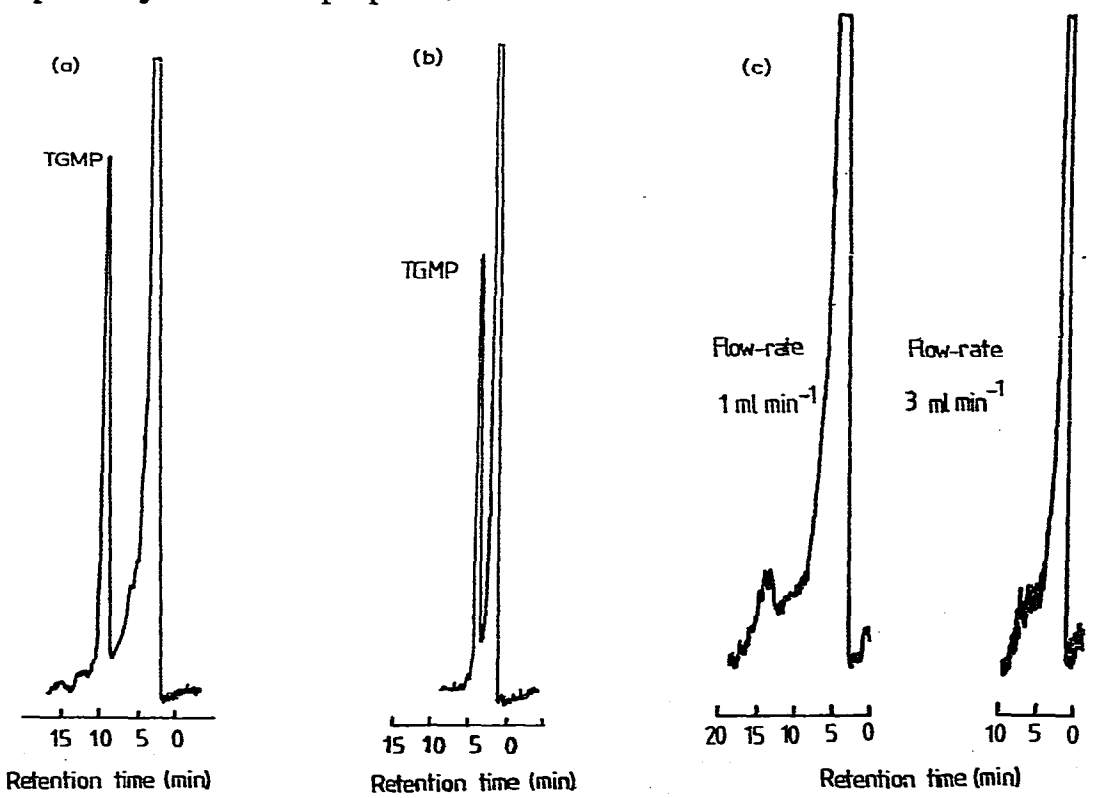

Fig. 3. Chromatograms of (a) an RBC extract using flow-fluorescent detection at a solvent flow-rate of 1 ml min^{-1}; (b) as a, except for flow-rate, 3 ml min^{-1}; (c) blank RBC extract at solvent flow-rates of 1 and 3 ml min^{-1}. Sensitivity of the fluorescence detector × 5 that in (a) and (b).

Deterioration of samples

Deterioration of the fluorophore was approximately 10% over 90 min and was not concentration dependent. For large numbers of samples, therefore, it is easier to oxidise (say) four samples shortly before injection.

Patients' samples stored at −20°C for periods from a few weeks to one year

were assayed and compared with the original results. Considerable degradation of the diphosphate to the monophosphate was seen after about three months. The monophosphate seemed to be stable up to six months.

Stock solutions in glass distilled, deionised water decayed by 10% at 4°C over a period of approximately 15 days. This was monitored and accounted for when plotting the peak height vs. RBC concentration curve by concurrently measuring an oxidised standard solution of TGMP prepared from the same stock solution used to spike the RBC samples. Regular preparation of fresh stock was impossible due to the small supply of TGMP solid.

The use of one stock solution made the monitoring of deterioration easier but resulted in a small dilution error when spiking RBC samples for analysis (see Determination of red blood cell TGMP concentrations).

With a mean standard deviation of ± 6.8% for all peak heights in the concentration range investigated (200—2000 ng ml^{-1}) reproducibility was thought to be high enough to obviate the necessity of an internal standard. By comparison with a standard solution of TGMP the isolation method was shown to be 100% efficient with no inhibition of oxidation by RBC constituents over the concentration range investigated. Factors such a injection error probably account for most of the error and to reduce this by using an internal standard would indeed improve the assay. However, after brief investigation, no suitable compound was found.

No interference was seen from oxidised 6-mercaptopurine added to TGMP extracts. Cytosine arabinoside and daunorubicin, drugs used with 6-thioguanine in leukaemia chemotherapy, do not show any measurable fluorescence at the wavelengths employed in this assay.

Excellent chromatographic separation of 6-thioguanine and its metabolites has been achieved [10] enabling the measurement of in vitro cellular levels of these compounds. Also, a highly reproducible, accurate assay for total 6-thioguanine nucleotides has been developed for the analysis of clinical RBC levels [7]. These methods however, employ solvent programming and multi-step separation techniques, respectively and for routine analysis, when time and economic considerations are important, these methods are probably unsuitable. The assay described here is sensitive and reproducible and its rapidity and simplicity of execution lend itself to routine usage.

RBCs are known to supply preformed purines to (amongst other tissues) the bone marrow [11] which is the main site of systemic toxicity of 6-thioguanine in mammals [12]. Following uptake, exogenous guanine is converted within mammalian RBCs to the nucleotide [13]. These factors make RBCs a useful model system for the design of an intracellular TGMP assay. The clinical relevance of RBC concentrations of TGMP has not been confirmed but might represent the magnitude of the supply of the active metabolite to the bone marrow cells. The measurement of leukaemic stem cell concentrations offers, perhaps, a more direct indication of drug effectiveness and indeed, clinical response has been associated with their magnitude using radiolabelled drug [14]. Studies are currently underway in our department to determine the relationship between RBC concentrations of TGMP and clinical response in leukaemic children treated with 6-mercaptopurine.

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