Fluorometric Assay of Thioguanine

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Abstract □ A spectrophotofluorometric method is described for the assay of thioguanine. The assay involves the oxidation of thioguanine with potassium permanganate to a fluorescent product. The method is suitable for routine quality control in laboratory preparations of thioguanine.

Keyphrases □ Thioguanine—spectrophotofluorometric assay □ Spectrophotofluorometry—determination, thioguanine

Basic quantitative data concerning drug dosage are required to obtain effective chemotherapy against tumor cells. Very sensitive and specific assays are needed to determine the anticipated low concentrations of anticancer drugs and their metabolites in various biological systems. Fluorometry, which affords the required sensitivity and specificity, has been used in this laboratory for such analytical determinations (1–5).

As part of an integrated program to investigate the pharmacokinetics and physiological disposition of antileukemic drugs, this laboratory became concerned with thioguanine (2-aminopurine-6-thiol), which inhibits a wide spectrum of neoplasms (6–9). In the initial studies, a quality control method was required for thioguanine injection solutions. Also, the method had to be adaptable for the assay of thioguanine in serum.

A literature survey revealed a limited number of applicable assays for thioguanine. One method employed thioguanine labeled with ^{35}S (10), whereas other assays used ^{14}C (7, 9). Although tracer methods generally extend the limits of sensitivity of an assay, they often lack specificity without prior separation of the drug from labeled metabolites. Pittillo and Woolley (11) reported a microbiological assay for thioguanine that had a lower limit of sensitivity of 1.0 μg/ml and required 24-hr preincubation and 16-hr incubation periods.

This report describes a simple and sensitive fluorometric assay for thioguanine standards which are oxidized with alkaline potassium permanganate solution to a fluorescent product. Fluorescence emission is measured at 410 nm with an excitation wavelength of 330 nm.

EXPERIMENTAL

Instrumentation—Fluorescence measurements were made with a spectrophotofluorometer[1] and an x–y recorder. The following instrument settings were used for all measurements: slit arrangement, No. 4; photomultiplier shutter, 4 mm; and sensitivity control, 40. Stainless steel mirrors were inserted in the cell compartment to increase the amount of radiation reaching the photomultiplier tube.

UV absorption measurements were made with a spectrophotometer[2].

Reagents—Combustion analyses for thioguanine[3] showed 35.9% carbon, 3.0% hydrogen, and 42.2% nitrogen. Calculated values were 35.9% carbon, 3.0% hydrogen, and 41.9% nitrogen. The UV absorption spectrum at pH 1 gave λ_{max} 257 (ϵ_m 8120) and 347 (ϵ_m 20,425) nm. These data agreed with previously reported values (12).

Thioguanine standards in water were prepared in a concentration range of 0.003–1.0 μg/ml from a 10-μg/ml stock solution. A drop of 6 N NaOH was added to 100 ml of the stock solution to dissolve the thioguanine.

Carbonate–bicarbonate buffer (pH 10.1) was prepared by the addition of 30.0 ml of 0.2 M sodium carbonate solution to 20.0 ml of 0.2 M sodium bicarbonate solution.

Potassium permanganate, 0.24% (w/v), and 30% hydrogen peroxide were also used.

Analytical Procedures—To 0.5 ml of pH 10.1 carbonate–bicarbonate buffer in a centrifuge tube was added 0.5 ml of thioguanine standard solution. After the buffered solution was stirred, 0.5 ml of 0.24% potassium permanganate was added to initiate oxidation of the drug. After 10 min of oxidation, the excess permanganate was reduced by the addition of 1 drop of 30% hydrogen peroxide. The resulting mixture was stirred thoroughly and centrifuged at 1640×g for 10 min. The clear, colorless supernate was measured fluorometrically at 410 nm when excited at 330 nm.

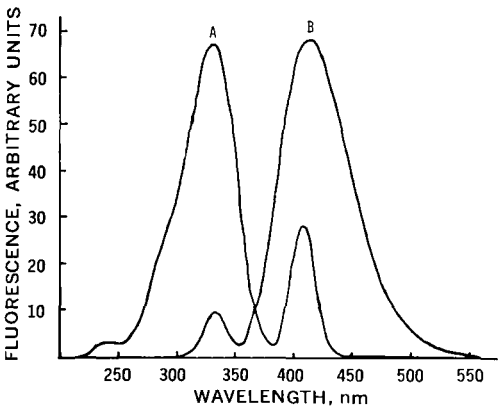

Figure 1—*Excitation spectrum (A) and fluorescence spectrum (B) of oxidized thioguanine (1.0 μg/ml).*

RESULTS AND DISCUSSION

Fluorescence Studies—The assay for thioguanine reported here is a modification of a fluorometric assay for mercaptopurine (1). Both compounds are susceptible to similar chemical changes. Since thioguanine does not have a high quantum yield of fluorescence, the drug was oxidized with alkaline potassium permanganate to a fluorescent product. The oxidation product was identified as 2-aminopurine-6-sulfonate.

[1] Aminco-Bowman.

[2] Cary model 17.

[3] Supplied by Dr. J. A. Montgomery, Organic Department, Southern Research Institute. All other purine derivatives used in this work were supplied by Dr. R. F. Pittillo, Microbiology Division, Southern Research Institute.

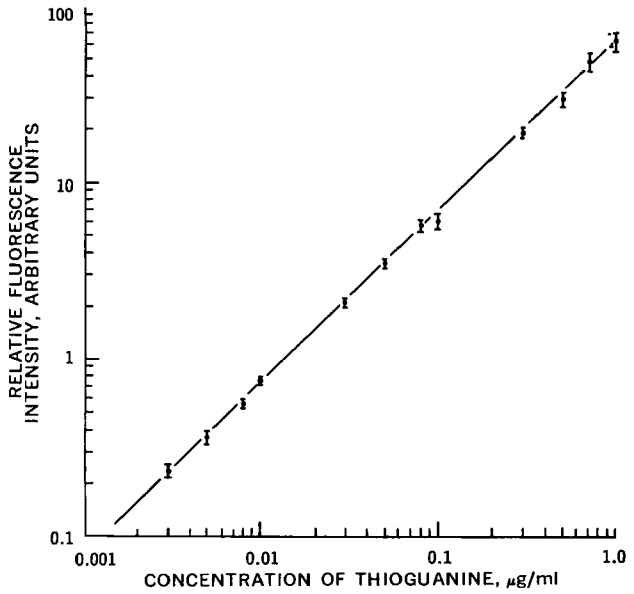

Figure 2—*Relative fluorescence intensities of thioguanine standards after potassium permanganate oxidation.*

The oxidations of thioguanine and mercaptopurine and their corresponding sulfinates with alkaline potassium permanganate to sulfonates were reported elsewhere (1, 13). Figure 1 shows the excitation and fluorescence spectra of thioguanine after oxidation. The maximum excitation occurs at 330 nm, and with that excitation wavelength the maximum fluorescence occurs at 410 nm.

The relative fluorescence intensity is linear with the concentration of thioguanine in water for the three concentration ranges examined (Fig. 2). The values plotted on the abscissa are concentrations of the thioguanine standards submitted to oxidation and do not include the 1 to 3 dilution of the 0.5-ml standard solution with buffer and permanganate.

Sensitivity—The lower limit of detection of thioguanine with the fluorometric assay is 0.003 μg/ml, whereas the upper limit is 1.0 μg/ml. Samples expected to have high drug concentration must be diluted. The average background relative fluorescence intensity of a water blank was 0.13 ± 0.02 arbitrary unit.

Reproducibility—Twenty samples containing 0.8 μg/ml of thioguanine were assayed by the fluorometric method. A set of 10 samples was measured in the morning, and a second set of 10 samples was determined in the afternoon. The mean value and standard deviation of the combined sets were 0.87 ± 0.07 μg/ml. The value of the afternoon samples was 0.84 ± 0.01 μg/ml, which indicates a better reproducibility as familiarity with the method and skills improved.

Interferences—To determine the specificity of the fluorometric assay for thioguanine, the fluorescence spectra of some related purine derivatives were examined. Table I presents the relative fluorescence intensity at 410 nm for each derivative oxidized and excited at 330 nm. Because of high fluorescence emission, two of the compounds were diluted to 1.0 μg/ml but all other purine derivatives were measured at a concentration of 10 μg/ml. Only one sample listed in Table I was not oxidized so as to observe differences of fluorescence intensities between unoxidized and oxidized thioguanine.

Table I—Relative Fluorescence Intensities of Purine Derivatives after Oxidation with Potassium Permanganate

Compound	Concentration, μg/ml	Relative Fluorescence Intensity
Thioguanine (unoxidized)	10	0.53
Thioguanine (oxidized)	1	62.49
6-Thioguanosine	1	49.20
Guanine	10	0.03
Xanthine	10	0.52
6-Thioxanthine	10	72.40
6-Thiouric acid	10	0.34

Although thioguanine did not strongly fluoresce, oxidation of the drug gave a product with over a 1000-fold increase in fluorescence intensity. Guanine, which serves as a starting material in the synthesis of thioguanine, produced the lowest relative fluorescence intensity and, thus, cannot interfere with this fluorometric assay. Only thioguanosine and 6-thioxanthine showed enough fluorescence to interfere; however, the presence of these two compounds in a thioguanine standard preparation is unlikely.

Although this fluorometric assay is presented for monitoring laboratory preparations of thioguanine, the assay with modifications is applicable to biological samples. Studies concerned with the measurement of thioguanine serum levels in mice, dogs, and monkeys were conducted in these laboratories. The *in vivo* studies will be reported.

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