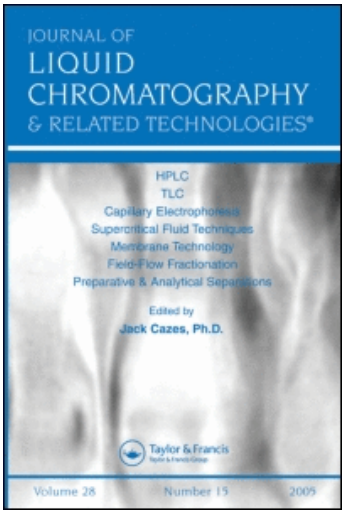

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A DUAL-COLUMN HPLC METHOD FOR THE SIMULTANEOUS MEASUREMENT OF 6-THIOGUANINE AND ADENINE IN RNA OR DNA

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ABSTRACT

A sensitive method for measuring 6-thioguanine incorporation into DNA and RNA utilizing a dual column system is presented. The measurement of the 6-thioguanine deoxyribo- or ribonucleosides and deoxyadenosine or adenosine is made simultaneously, thereby allowing for direct calculation of the incorporation per nucleic acid base. The separation utilizes a strong anion-exchange column connected in series with an octadecylsilane column. Prior to high pressure liquid chromatography, the sample is partially purified and oxidized with potassium permanganate. Following a 10-min delay, a 10-min linear gradient from 2% to 20% methanol in 30 mM $NH_4H_2PO_4$, pH 3.7, is employed. Detection of eluting material is by fluorescence and by UV absorbance at 254 nm. Recovery of the 6-thioguanine nucleosides was determined using [8-^{14}C]-6-thioguanine. The sensitivity of the method for the oxidized 6-thioguanine compounds is approximately 1 pmole (fluorescence) whereas that for the adenine nucleosides (UV absorbance) is about 100 pmoles. This sensitivity is adequate to determine the incorporation in less than 10^6 (about 1 mg) Chinese hamster ovary cells exposed to a cytotoxic concentration of 6-thioguanine.

INTRODUCTION

Since its introduction more than 10 years ago for the measurement of biological components and drugs, high pressure liquid chromatography (HPLC) has been considerably improved.

Among the first applications of HPLC to biological samples was the work by Brown (1) in the laboratory of Dr. R. E. Parks, Jr., which described the analysis of purines and pyrimidines using pellicular ion exchange columns. Currently, a simple and improved separation of purine and pyrimidine nucleotides can be achieved using a commercially available microparticulate anion exchange column (2). Likewise, chemically bonded, reversed-phase columns can now be used to efficiently separate purine and pyrimidine nucleosides and bases (3,4). The method presented herein takes advantage of both columns, coupled in series, for the simultaneous measurement of 6-thioguanosine (TGR) or β-2'-deoxythioguanosine (βTGdR) and adenosine (Ado) or deoxyadenosine (dAdo). Alkaline permanganate oxidation of 6-thioguanine (6-TG) derivatives yields compounds that are highly fluorescent and are retained by the anion-exchange column (5). Following the isocratic elution of the oxidation products from the anion-exchange column, Ado or dAdo are removed from the reversed-phase column using a methanol gradient. Thus, in the same sample the amount of 6-TG incorporated into RNA or DNA can be compared to the adenine base content. This procedure corrects for differences between experiments in the extent of RNA or DNA extracted and hydrolyzed, analogous to the advantages conferred by the use of an internal standard.

MATERIALS

6-TG and βTGdR were provided through the courtesy of the Drug Synthesis and Development Branch, National Cancer Institute,

National Institutes of Health, Bethesda, MD. The [8-^{14}C]-6-TG (55 μCi/μmole) was synthesized by Moravek Biochemicals, Brea, CA, and found to be 90% pure by HPLC. Ammonium dihydrogen phosphate, perchloric acid, potassium hydroxide, trichloroacetic acid, hydrochloric acid, sodium carbonate, sodium bicarbonate, potassium permanganate, glycine, and hydrogen peroxide were purchased from Fisher Scientific Co., Pittsburg, PA. Magnesium chloride, TGR, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), deoxyribonuclease I, 5'-nucleotidase, alkaline phosphatase, and snake venom phosphodiesterase were obtained from Sigma Chemical Company, St. Louis, MO. HPLC-grade methanol came from Burdick and Jackson Laboratories, Inc., Muskegon, MI. RPMI 1640 was purchased from Gibco, Grand Island, NY, and fetal calf serum was obtained from K.C. Biologicals, Lenexa, KS.

The HPLC system was a Laboratory Data Control (Riviera Beach, FL) Series 7800 Liquid Chromatograph operated by a Chromatography Control Module II (CCM). A Rheodyne model 7120 injector (Rheodyne Inc., Berkley, CA), Partisil-10 SAX column (4.6 mm x 25 cm, Whatman, Inc., Clifton, NJ), and μBondapak C_{18} column (4 mm x 30 cm, Waters Associates, Milford, MA) were used. Eluting materials were detected by ultraviolet absorbance at 254 nm (UV III Monitor, Laboratory Data Control) and by fluorescence (Schoeffel Model FS-970 Spectrophoto-fluorometer, Schoeffel Instruments, Westwood, NJ). The outputs of the two detectors were plotted and integrated simultaneously by the CCM, using the machine language programmed features of the instrument. Statistical calculations were

performed using the BASIC programmable features of the CCM microprocessor, as supplied by Mr. Mike Tarter of Laboratory Data Control.

METHODS

Chinese hamster ovary (CHO) cells were maintained in continuous culture by passge in RPMI 1640 medium containing 20% fetal calf serum and antibiotics (penicillin and streptomycin, 100 units/ml; amphotericin, 0.25 μg/ml). Cells were grown in the presence of various levels of 6-TG for 24 hr prior to harvest. The cells were then treated with trypsin and washed with 0.14 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. The cells ($5x10^7$) were concentrated by centrifugation at 100 xg. Subsequent procedures were performed at 4°C, and centrifugation was at 1000 xg for 10 min. The cells were extracted with 1.0 ml of 0.8 N perchloric acid and the sample was centifuged. The supernatant, which contained the acid-soluble (nucleotide) fraction, was neutralized with 10 N potassium hydroxide and stored at -20°C. The acid-insoluble pellet was incubated overnight at room temperature in 1 ml of 0.4 N potassium hydroxide to hydrolyze the RNA. DNA was then precipitated with cold 4 N hydrochloric acid (0.1 ml) and 50% trichloroacetic acid (0.3 ml). After centrifugation, the supernatant (hydrolyzed RNA) was neutralized and treated with 0.3 ml of a solution containing 0.15 units of alkaline phosphatase, 100 mM glycine, and 50 mM magnesium chloride, pH 8.0. The sample was then incubated for 3 hr at room

temperature to hydrolyze the 2' and 3' nucleotides. Protein was denatured by placing the tubes in a boiling water bath for 2 min. The sample was then centrifuged and the supernatant was stored at -20°C. The DNA was hydrolyzed by incubation at room temperature for 16 hr with deoxyribonuclease I (145 Kunitz units), 5'-nucleotidase (0.25 units), and phosphodiesterase (0.015 units), in 1 ml of 0.1 M Tris-HCL and 0.1 M magnesium chloride, pH 8.0. Reprecipitation of the protein and lipid was accomplished with the addition of 0.5 ml of cold 50% trichloroacetic acid. The sample was then centrifuged. The supernatant, containing the hydrolyzed DNA, was neutralized with 10 N potassium hydroxide. Following the method of Tidd and Dedhar (5), 0.1 ml of the hydrolyzed DNA or RNA was incubated with 0.1 ml of 0.1 M sodium carbonate-sodium bicarbonate buffer, pH 10.1, and 0.1 ml of 0.24% potassium permanganate. The oxidation was allowed to proceed for 5 min at room temperature. Reduction of the excess permanganate was achieved by the addition of 10 µl of 30% hydrogen peroxide, and the resultant manganese dioxide precipitate was removed by centrifugation at room temperature. The oxidized samples were then analyzed by HPLC using the Partisil-10 SAX column with its outlet coupled to the inlet of the µBondapak C_{18} column. After injection of the sample, the oxidized thiopurines were eluted from the Partisil SAX column using 30 mM ammonium dihydrogen phosphate, pH 3.7, plus 2% methanol. Ten min after injection, a linear gradient to 30 mM ammonium dihydrogen phosphate, pH 3.7, plus 20% methanol was formed in 10 min. The flow rate was 2 ml/min. The

eluting materials were monitored by UV absorbance at 254 nm (0.032 AUFS) and by fluorescence. The Schoeffel FS 970 Detector settings were: excitation wavelength, 310 nm; emission filter, 389 nm cut-off; entrance filter, Corning 7-54; time constant, 4 sec; range, 0.5; and sensitivity, 90.0.

RESULTS

A representative example of the simultaneous measurement of βTGdR and dAdo in a DNA hydrolysate from CHO cells is presented in Figure 1. The fluorescent detector output is shown from the time of injection until ~15 min; thereafter, the UV absorbance at 254 nm is plotted. The 2% to 20% methanol gradient beginning at 10 min after injection is also shown. The oxidized product of βTGdR eluted at 14-15 min (fluorescence), whereas dAdo eluted following the methanol gradient at 22 min. The identity of the oxidized βTGdR was confirmed by comparison of its retention time to that of an authentic standard; the presence of the fluorescent peak at 14-15 min required permanganate oxidation; and the observation that identical extracts of cells incubated in the absence of 6-TG (control) did not contain the fluorescent component. The identity of dAdo was confirmed by its retention time and sensitivity to adenosine deaminase. Peaks other than the above were not identified; however, the small fluorescent peak at about 13 min may be the oxidized derivative of 6-TG, since the retention time agrees with that of standards, and it was only present in cells incubated with 6-TG.

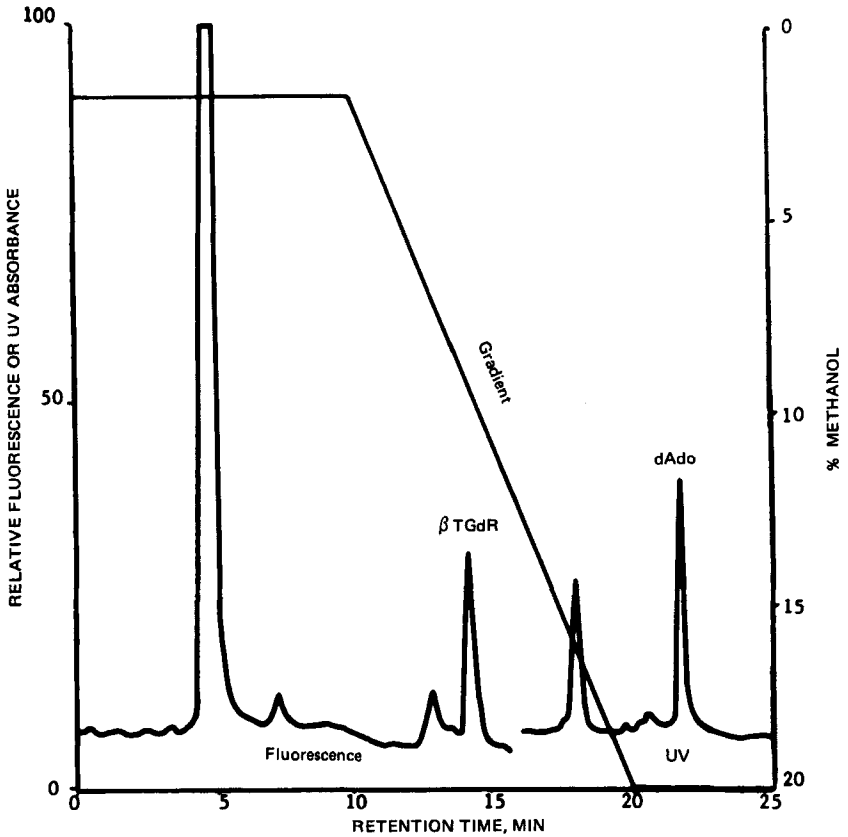

Figure 1. Simultaneous determination of βTGdR and of dAdo in a DNA hydrolysate of CHO cells. The DNA of approximately 1.8×10^7 CHO cells, which had been exposed to 6-TG (0.5 μg/ml) for 24 hr, was enzymatically converted to its 2'-deoxyribonucleosides. The sample was then subjected to alkaline permanganate oxidation as described by Tidd and Dedhar (5). The oxidation generates a highly fluorescent derivative of βTGdR. The oxidation product was detected by fluorescence after elution from a Partisil SAX column with 30 mM $NH_4H_2PO_4$, pH 3.7. dAdo was then eluted from a μBondapak C_{18} column (coupled in series to the Partisil SAX column) by the methanol gradient shown. dAdo was detected by UV absorbance at 254 nm. In the sample shown, 7 pmoles of βTGdR and 0.75 nmoles of dAdo were present.

A representative separation of the oxidized derivative of TGR and Ado in a RNA hydrolysate from CHO cells is presented in Figure 2. The detector outputs and gradient are plotted analogous to that discussed above for Figure 1. The oxidized derivative of TGR was not present in cells incubated in the absence of 6-TG. The component co-eluted with authentic, oxidized TGR, and this fluorescent component was present only in samples treated with permanganate. Identity of Ado was confirmed by its retention time and sensitivity to adenosine deaminase. A criticism of acid extraction techniques has been that a high degree of mutual cross contamination of DNA and RNA occurs (6). However, as illustrated in Figures 1 and 2, there are non-detectable amounts of oxidized derivative of TGR or Ado in the DNA extract, and correspondingly small amounts (if any) of βTGdR or dAdo in the RNA extracts.

When the oxidation products of TGR in RNA or βTGdR in DNA are measured, there is considerable variation from one experiment to another (Table 1). For example, the amount of βTGdR measured per 10^6 cells varied from 7.5 to 136.5 pmoles in the three experiments shown in Table 1. This variability may be due to differences between experiments in the efficiency of the enzyme digestion of DNA, since there was a similar variation in the measurement of dAdo (Table 1). That is, the ratio of βTGdR to dAdo corrects, to a large measure, this high degree of variation. The ratio ranged from 2.95 to 6.01 in the three experiments. Similar, though less extensive, variations between experiments were observed in the measurements of TGR and Ado in RNA hydrolysates. The greater

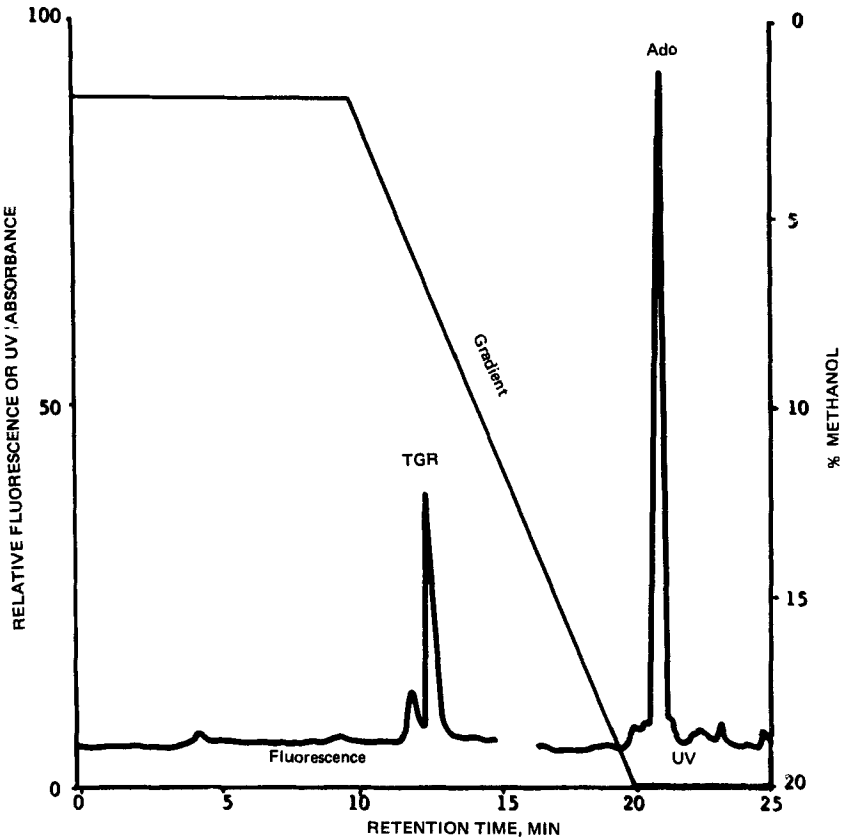

Figure 2 Simultaneous determination of TGR and of Ado in a RNA hydrolysate of CHO cells. The RNA of approximately 1.8 x 10^7 CHO cells, which had been exposed to 6-TG (0.5 µg/ml) for 24 hr, was enzymatically converted to its ribonucleosides. The sample was then subjected to alkaline permanganate oxidation as described by Tidd and Dedhar (5). The oxidation generates a highly fluorescent derivative of TGR. The oxidation product was detected by fluorescence after elution from a Partisil SAX column with 30 mM $NH_4H_2PO_4$, pH 3.7. Ado was then eluted from a µBondapak C_{18} column (coupled in series to the Partisil SAX column) by the methanol gradient shown. Ado was detected by UV absorbance at 254 nm. There were 10 pmoles of TGR and 1.8 nmoles of Ado present in the injected sample.

TABLE 1

Incorporation of 6-TG into the DNA and RNA of CHO Cells

Experiment	βTGdR Incorporation pmol/10^6 cells	dAdo pmol/10^6 cells	pmol βTGdR per 100 pmol dAdo
		DNA	
1	31.8	883	3.60
2	136.5	2272	6.01
3	7.5	254	2.95
mean ± S.E.	58.6 ± 39.6	1136 ± 596	4.19 ● 0.93
		RNA	
Experiment	TGR Incorporation pmol/10^6 cells	Ado pmol/10^6 cells	pmol TGR per 100 pmol Ado
1	321	23257	1.38
2	228	11628	1.96
3	107	7172	1.49
mean ± S.E.	219 ± 62	14019 ± 4763	1.61 ± 0.18

Approximately 5 x 10^7 CHO cells were exposed to 6-TG (50 μg/ml) for 24 hr, at which time the cells were harvested. The DNA and RNA were extracted and hydrolyzed to the deoxyribo- and ribonucleosides as described by Tidd and Dedhar (5). 6-TG incorporation was measured by fluorescence of the oxidation products of βTGdR and TGR. dAdo and Ado were measured by UV absorbance at 254 nm using the HPLC method illustrated in Figures 1 and 2.

reproducibility of the measurement of TGR in RNA may relate to a higher degree of reproducibility of the alkaline hydrolysis of RNA, or it may be due to other factors. Utilization of the ratio of TGR in RNA to the endogenous adenine (Ado in Table 1) further reduces the variance.

The recovery of 6-TG in the RNA and DNA hydrolysates was determined using radioactive 6-TG (Table 2). The extraction technique generates acid-soluble and acid-insoluble fractions. The acid-insoluble fraction is treated with potassium hydroxide and alkaline phosphatase to hydrolyze RNA, in which TGR is measured as shown in Figure 2. In the RNA hydrolysates, TGR

TABLE 2

Recovery of [8-^{14}C]-6-TG During Extraction, Hydrolysis and Analysis by HPLC

Sample	nmol/2 x 10^7 cells
Acid-soluble Fraction	0.23 ± 0.10
Acid-insoluble Fraction	
RNA hydrolysate	0.70 ± 0.08
TGR (RNA)	0.33 ± 0.07
DNA hydrolysate	0.41 ± 0.18
βTGdR (DNA)	0.29 ± 0.14
Other (Protein, Lipid)	0.16 ± 0.11

Cells were incubated with 0.5 μg/ml of [8-^{14}C]-6-TG (55 μCi/μmole) for 24 hr as described in Methods. The cells were extracted and nucleosides were prepared from the RNA and DNA. The amounts of TGR and βTGdR were determined from radioactivity in the HPLC fractions corresponding to their oxidation products (Figures 1 and 2). Mean values ± S.E. are shown, n=4.

accounted for 47% of the radioactivity present, i.e., 0.33 of the total 0.70 nmoles. Whether this recovery represents effects of the extraction, hydrolysis, and oxidation procedures on the chemical stability of the 6-TG moiety or is indicative of base modifications of the 6-TG in the RNA is not known. Recovery of βTGdR in the DNA hydrolysates averaged 71%, i.e., 0.29 of the 0.41 nmoles. The HPLC and radioisotopic determinations of TGR and βTGdR in these experiments were in good agreement (Table 3).

DISCUSSION

The incorporation of 6-TG and 6-mercaptopurine into cellular DNA and RNA as 6-TG nucleotide has been shown by several investigators (7). Metabolites of these 6-thiopurines inhibit purine biosynthesis de novo and inhibit enzymes involved in the interconversion of purine nucleotides. Incorporation into DNA correlates well with cytotoxicity in most cells; however, the exact mechanism for the toxicity is not known. Incorporation has been determined by radioisotopic techniques using ^{14}C- or ^{35}S-labeled drug and by the fluorometric method of Tidd and Dedhar (5). The latter technique involves oxidation of 6-TG or its derivatives to highly fluorescent compounds (8). The oxidized products, which are anions, can then be separated using an anion-exchange column (5). The method reported herein is a modification of that of Tidd and Dedhar and permits the simultaneous determination of endogenous adenine in RNA or DNA, providing a means to correct for differences in recovery, hydrolysis, etc.

TABLE 3

Determination of 6-TG Incorporation into Nucleic Acids of CHO Cells

	βTGdR in DNA pmoles/sample		TGR in RNA pmoles/sample	
Experiment	Fluorescence	Radioactivity	Fluorescence	Radioactivity
1	28	48	369	387
2	159	116	238	269
3	870	660	271	170
4	337	344	318	497

The cells were incubated with [8-^{14}C]-6-TG as described in Table 2. βTGdR and TGR were measured in hydrolysates of RNA and DNA as shown in Figures 1 and 2; they were also measured by radioactivity associated with their oxidation products as described in Table 2. The results shown are single determinations for four separate experiments.

between samples. To measure the fluorescent derivatives of βTGdR or TGR, and dAdo or Ado in the same sample, we coupled an anion exchange column with a reversed-phase column. The reversed-phase column has been used as an efficient tool for separating nucleosides and bases (3,4); however, in the absence of an ion-pairing reagent, the oxidized 6-TG derivatives are not retained. We did not attempt to use an ion-pairing reagent, since the coupling of the two columns provided sufficient resolution (Figures 1 and 2). By simultaneous measurement of endogenous adenine, the variance between samples is reduced markedly (Table 1). Experiments utilizing [8-^{14}C]-6-TG indicated that the acid-extraction method gave good recovery of 6-TG in RNA and DNA (Table 2). Furthermore, the fluorometric HPLC method agreed well with the radioisotopic measurement of 6-TG in DNA and RNA (Table 3). Although not attempted by us, a less drastic procedure to purify macromolecules containing 6-TG using organomercurial agarose has been reported (9).

The dual column system may have additional utility beyond the specific purpose reported herein. For example, the Partisil SAX column provides rapid, facile separation of purine and pyrimidine nucleotides (2). It may be feasible, therefore, to measure nucleosides, bases and nucleotides in the same injected sample by using the coupled columns. Such columns are currently being used sequentially for this purpose (10). The Partisil SAX column has been used to remove nucleotides from biological samples prior to analysis of nucleosides and bases by reversed-phase chromatography

(11). Alternatively, it may be possible to combine cation and anion HPLC columns for this purpose (12), similar to the use of mixed-bed resins for amino acid analysis. The use of dual columns may allow for separations not currently feasible with only one column, thus expanding the capabilities of HPLC and decreasing analysis time.

ACKNOWLEDGEMENT

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