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Biological Monitoring Screening of Patients Provided Antineoplastic Drugs Including Adriamycin, Cyclophosphamide, 5-Fluorouracil, Methotrexate, and Vincristine

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ABSTRACT

The aim of the present study was to establish screening biomarkers of exposure to antineoplastic drugs administered to 11 patients undergoing cancer chemotherapy. Among the anticancer drugs administered were cyclophosphamide (all), Adriamycin (5 of 11), methotrexate (3 of 11), 5-fluorouracil (4 of 11), vincristine (3 of 11), megestrol acetate (1 of 11), and procarbazine (1 of 11). The noninvasive urinary parameters investigated were thioethers, D-glucaric acid, elements, and forward and reverse mutagenesis using bacterial bioassays. The data were analyzed in terms of the observed concentrations and those corrected for personal baseline. Personal baseline correction for parameters with significant nonexposure baseline levels was essential. While glucaric acid and thioethers were increased by the drug treatments, the correlations with baseline-uncorrected data showing an inverse relationship proved spurious, because saturation of the detoxification systems occurred at the high doses administered. Glucaric acid was also influenced by methotrexate and vincristine. Thioether content was affected by cyclophosphamide only. The forward mutagenesis assay was directly correlated to cyclophosphamide dose but the reverse assay was not, in the presence or absence of rat S9 fraction. The forward assay was not sensitive to the effects of smoking. Relative to controls, the elements changed by cyclophosphamide were K, S, and P. Those affected by Adriamycin were Ca, Mg, and Na; 5-fluorouracil affected Ca, Mg, Na, and C; methotrexate changed P and S. The forward mutagenesis assay and D-glucaric acid concentrations were the screening biomarkers best suited to monitoring for extent of exposure to these antineoplastic drugs.

INTRODUCTION

The use of chemicals to treat cancer (cancer chemotherapy) began near the end of World War II with the discovery of the antitumor properties (1) of nitrogen mustard and its derivatives. Anticancer drug administration has increased greatly (2). Some 60 drugs are currently commercially available, and these are administered to an estimated 200,000–400,000 patients annually (3–5). The present study was concerned primarily with CP[3] and ADR and the anticancer drugs co-administered to patients with them.

Many antineoplastic agents are carcinogens and teratogens in experimental animals (6–8). The International Association for Research on Cancer has classified CP, chlorambucil, and 1,4-butanediol dimethylsulfonate as human carcinogens (2, 6). CP is also a human teratogen (7). ADR (doxorubicin) is an animal carcinogen (2, 6). Nurses or pharmacy technicians who handle these drugs may show the symptoms of anticancer drug overexposure, as may patients (9); some of these effects include anemia, thrombocytopenia, neutropenia, and immunodeficiency. Often, agents with different mechanisms of action are combined to avoid the development of resistance to any single agent (10). For example, CP, ADR, and 5-FU are frequently administered in combination to provide effective therapy.

CP is unable to act as an alkylating agent until it is metabolized by microsomal mixed function oxidases (7, 11). Jardine *et al.* (12) showed that approximately 35% of an injected dose of CP can be recovered as parent compound or the metabolites phosphorodiamidic acid mustard and nornitrogen mustard in urine within 24 h of injection, most within the first 4 h with very little in the last 4 h. CP was excreted in the urine by 12 h and nornitrogen mustard peaked at 12–16 h. Colvin and Hilton (13) showed that 70–85% of a CP dose was accounted for in animals by the urinary metabolites carboxyphosphoramide and 4-ketocyclophosphamide, neither of which was measured by Jardine *et al.* The plasma half-life of CP is 3.5–7.0 h (13).

ADR binds to DNA mostly by intercalation of the aglycone and electrostatic interactions between the sugar/phosphate backbone of DNA and the glycosidic side chain of ADR (14, 15). The major metabolites of ADR, adriamycinol and Adriamycin aglycone, are cleared primarily (60%) through biliary excretion, with urinary excretion accounting for less than 10% of the clearance of ADR (14). The aglycone is conjugated to sulfate or glucuronic acid, and these conjugates are excreted in the urine and bile (16). The elimination phase of ADR has been reported to be approximately 17 h in patients with normal liver function; 41% of the administered dose was detected in 7 days by analysis of biliary levels (14).

CP can be absorbed percutaneously (12). Skin absorption in nurses and pharmacists has been noted after direct contact via spillage during preparation or administration or after contact with excreta of patients undergoing therapy (17). Due to the variety of drugs handled, the innumerable metabolites formed, and the expected low concentrations present, analytical methods for determining blood and urine concentrations of each individual drug are not attractive economically, although methods have been developed for cisplatin (18) and urinary CP (17) for oncology nurses.

Biological monitoring levels reflect exposure from all routes (19, 20). The levels in biological media are affected by individual metabolizing and dermal penetration capabilities, type of chemical, use of protective garments and gloves, individual work habits, manipulating skills, rate of breathing, and work exertion.

Nonspecific biological monitoring screening tests like measurements of serum enzymes (21), urinary porphyrins (22), D-glucaric acid (23, 24), mutagens (25, 26), or thioethers (27) reflect the net effect of exposure to chemicals but are not necessarily related to the dose of a single agent (28). Such nonspecific tests are most useful in monitoring exposure to mixtures of compounds, as in the case of oncology nurses and

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[3] The abbreviations used are: CP, cyclophosphamide; ADR, Adriamycin; DMSO, dimethyl sulfoxide; GA, D-glucaric acid; 5-FU, 5-fluorouracil; MTX, methotrexate; VIN, vincristine; MEG, megestrol acetate; THIO, thioethers; CR, creatinine; PRO, procarbazine; *df*, degrees of freedom.

pharmacists handling many different drugs or of patients receiving many drugs. Urinary THIO and GA concentrations are nonspecific measures of the metabolizing capability of the liver and kidney for chemical compounds. High levels do not necessarily indicate damage to the organism or to the liver or kidney, but rather a metabolic and clearance response by the liver or kidney to potentially toxic materials. Critical lower and upper thresholds might exist that are related to adverse effects.

Biological monitoring techniques are needed which are economically feasible and capable of detecting exposure to a variety of antineoplastic drugs, since combination therapy is invariably followed. The present study sought to examine the utility of THIO, GA, metals, and mutagenic activity as nonspecific biological monitoring screening parameters of urine correlated to exposure to antineoplastic drugs in patients undergoing chemotherapy.

MATERIALS AND METHODS

Eleven cancer patients being treated with CP or ADR and other coadministered anticancer agents were recruited from the Oncology Department at the University Hospital (Cincinnati, OH), through the cooperation of the practicing oncologists Dr. Deborah Green and Dr. Andrew Denton. Additional samples were obtained from the Oncology Clinic at Fort Hamilton Hughes Hospital (Hamilton, OH), through the auspices of Dr. Weisenberger, Department Director. The cancer patients had non-liver or non-kidney cancers and showed no evidence of liver or renal dysfunctions.

Fifteen control subjects were also selected; they had no extraordinary exposure to mutagens through occupation or medication. All subjects signed consent forms and completed questionnaires regarding present and prior antineoplastic treatment doses, any drug side effects, cancer type, and surgery history. Data were also collected on previous radiation treatments, other medications, age, gender, height, weight, smoking history, and diet.

Sample Collection and Storage

All subjects provided a 24-h urine sample. For the chemotherapy patients, collection began immediately after drug administration. A spot urine sample just prior to drug administration was also obtained.

The samples were collected in acid-washed 1-gallon polyethylene containers. The urine was either stored on ice, kept in a dark bag under snow, or refrigerated during collection. The samples were thawed, mixed thoroughly in the laboratory by manual shaking, the volume was measured, and samples were filtered through a Whatman No. 1 filter. Some samples were stored unfiltered. Aliquots of approximately 125 ml (for mutagenesis studies) or 10 ml (for chemical tests) were distributed into sterile metal-free containers and stored at −20°C. This allowed the urine sample to be analyzed further with only one more freeze/thaw, instead of the many cycles which could result in deterioration.

Safety

The precautions for handling suspect carcinogens included: weighing in a glove box, use of a laminar flow hood for mutagenicity testing, and use of proper protective clothing and equipment such as disposable gloves, gowns, and masks. Appropriate disposal was also followed. Reusable glassware was washed with laboratory cleanser, rinsed several times with water, and then rinsed 3 final times with deionized water. Glassware was then soaked overnight in 10% nitric acid, rinsed 3 times with distilled water, and dried in a dustless oven.

Chemical Parameters

Urinary Creatinine Determinations. Urine CR levels were determined on aliquots of thawed urine samples, using the alkaline picrate method (29) as automated by DuPont on their Automatic Chemical Analyzer. The inter- and intrarun coefficients of variation reported by DuPont are 0.6% and 1.8%, respectively.

The concentrations of all the chemical measures were divided by the corresponding CR concentration to obtain the appropriate normalized measure. The daily excretion rate was also calculated.

D-Glucaric Acid Assay. Limpet β-glucuronidase type L-II lyophilized powder (890,000 Sigma units/g), D-saccharic acid monopotassium salt, glycine sodium salt (sodium glycinate), and phenolphthalein mono-β-glucuronic acid sodium salt from rabbit urine were purchased from Sigma Chemical Company (St. Louis, MO). Sodium acetate, glacial acetic acid (99.7%, w/w), hydrochloric acid (37%, w/w), and sodium hydroxide (98.4% pure) were obtained from Fisher Scientific Company (Pittsburgh, PA).

The following working solutions were used: 0.5 M acetate buffer, pH 3.8, and 3 M glycine buffer, adjusted to pH 11.5. Both buffers were stored at 4°C.

The working enzyme solution (β-glucuronidase, 250 Sigma units/ml in deionized water) and the substrate (0.5 mM phenolphthalein mono-β-glucuronic acid in 0.5 M acetate buffer at pH 3.8) were prepared fresh daily.

The optimized procedure, based on one by Colombi *et al.* (23), is as follows. Frozen urine samples were thawed at room temperature, thoroughly mixed, and centrifuged (1500 rpm, 5 min, room temperature). Aliquots (0.2 ml) of the supernatant were placed in 20-ml glass scintillation vials. Seven and one half ml 0.5 M acetate buffer, pH 3.8, were added and mixed well and the solution was placed in a boiling water bath for 30 min. After cooling, a 2.0-ml aliquot was added to a 16- x 125-mm glass test tube. To this test tube, 0.2 ml of the substrate (0.5 mM phenolphthalein mono-β-glucuronic acid in 0.5 M acetate buffer) was added, and the mixture was placed in a 37°C water bath for 10 min. The reaction was started by adding 0.2 ml of the working enzyme solution to the test tube in the water bath. After exactly 30 min, the reaction was stopped by adding 3 ml 3 M glycine buffer, pH 11.5. The absorbance was read at 550 nm in a 1-cm cell, between 5 and 15 min, using a Gilford Model 240 spectrophotometer. In each assay, D-saccharic acid monopotassium salt (potassium hydrogen glucarate) was used as a reference standard and was carried through the same procedure. The GA concentration was calculated by comparison to this standard curve. For quality control, an aliquot from a reference urine sample was included in each assay. An assay was rejected if the GA determination of the reference urine sample did not lie within ±2 SD from the cumulative sample arithmetic mean.

All samples were assayed in at least two independent measurements, using triplicate determinations in most cases. For those samples having a coefficient of variation of >30%, additional assays were performed and, if necessary, a result was rejected if outside the 90% confidence limits as shown by the Q test (30). All corresponding coded samples were analyzed together in an investigator-blind manner. The order of the samples within each assay was randomized.

Thioether Assay. 5,5′-Dithiobis-(2-nitrobenzoic acid) and reduced glutathione were purchased from Sigma; hydrochloric acid (37%, w/w), sodium hydroxide (98.4% pure), sodium chloride (Certified ACS), ethyl acetate (Pesticide Grade), and methyl acetate (Certified Grade) were from Fisher Scientific Co. Monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) was obtained from Mallinckrodt Chemical Works (St. Louis, MO).

The working solutions were 0.1 M phosphate buffer, pH 8, and an Ellman's reagent that was prepared daily by adding 69 ml of stock Ellman's reagent [0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer] to 31 ml 0.1 M phosphate buffer.

The optimized procedure was based on one by Igwe *et al.* (31). Frozen urine samples were thawed at room temperature and the urine was thoroughly shaken and centrifuged (1500 rpm using a Sorvall II general laboratory centrifuge, 5 min, room temperature). Aliquots (3.0 ml) of the supernatant were placed in 16- x 125-mm screw cap glass test tubes. Two hundred μl 4 M HCl and 0.5 g NaCl were added and mixed well. After standing at room temperature for 15 min, 7.5 ml of ethyl acetate were added and the solution in the test tube was shaken vigorously for 15 min. The test tube was centrifuged (1500 rpm, 5 min, room temperature), and the ethyl acetate layer was transferred by Pasteur pipet to a clean 20-ml glass scintillation vial. A second similar extraction was performed, and the second ethyl acetate extract was

added to the first. The combined extract was evaporated just to dryness under a stream of nitrogen on a water bath (40 ± 3°C). The residue was taken up in 1.0 ml of Ellman's reagent. The concentration of —SH groups was calculated from the corrected absorbances, using a 1-cm cell in a Gilford Model 240 spectrophotometer and a molar absorptivity of 13.6 nM^{-1} cm^{-1}. Reduced glutathione was used to determine the molar absorptivity for the —SH determination. Alkaline hydrolysis was then carried out on a 0.4-ml aliquot of the ethyl acetate extract, in a glass screw cap test tube, by adding 200 μl 4 M NaOH, saturating with nitrogen, capping, and incubating the mixture in a boiling water bath for 50 min. After cooling, the mixture was neutralized with 200 μl 4 M HCl. To this mixture, 4.2 ml of working solution of Ellman's reagent was added. The sulfhydryl concentration was determined as described above. THIO concentrations were expressed as mmol —SH/mol creatinine. For quality control, an aliquot from a reference urine sample was included in each assay, as for the GA assay.

Multielemental Analyses. Thawed urine samples were analyzed for 34 elements by simultaneous inductively coupled plasma atomic emission spectrometry, using the 1:6 dilution technique (32). The analytical wavelengths, quality assurance and control, Jarrell Ash/Allied Chemical ICAP-9000 vacuum instrument, analysis conditions, reagents, and cleaning procedures have been described in detail elsewhere (33). The minimum quantifiable concentrations for these samples, using modified *k* factors, were, in μg/ml; 5–10 Cl; 1–5 C; 0.1–1 I, In, and Sn; 0.05–0.1 As, B, K, and Tl; 0.01–0.05 Ag, Hg, Na, P, Pb, S, Se, and Zn; 0.005–0.01 Au, Ba, Cr, Fe, Ni, Sb, and Si; 0.001–0.005 Al, Ca, Cd, Co, Cu, Mg, Mn, Mo, and Pt; and <0.001 Be, Li, Sr, Ti, and V. An (*N* + 1) scanner was used for analysis of carbon and Cl.

Mutagenicity Assays. The chemicals used were: acetone, methanol, and methylene chloride (all Pesticide Grade) purchased from Fisher Scientific Co. and DMSO purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI).

Sulfatase (29,000 units/g) and β-glucuronidase from bacteria (1,000,000 units/g) were purchased from Sigma. XAD-2 residue-free 20/50 mesh resin was purchased from Alltech Associates (Deerfield, IL).

The procedures required to attain the optimum concentration and detection of the mutagens in urine are described in detail elsewhere (34). The XAD-2 resin was cleaned by placing it in a cellulose thimble and extracting with 200 ml methanol by soxhlet extraction for 2 h, followed by a similar extraction with 200 ml acetone for 2 h. To prepare the columns, a glass wool plug was inserted at the base of the column, the slurry of resin was loaded onto the column (1.5-cm diameter and 5- or 16.3-cm height), and the column was hand vibrated to exclude air bubbles. A layer (6 mm) of cleaned sand (acetone-, methanol-, and water-rinsed Ottawa Sand) was added to the top of the column. The column was rinsed with 600 ml deionized water and stored at −4°C until used.

A volume of 100 ml of thawed urine was loaded onto the wet slurry column of XAD-2 resin (in a column 1.5 cm x 16.3 cm) to maintain a flow rate of 1–2 ml /min. The column was rinsed with 100 ml of water. Compounds retained by the column were then eluted with 50 ml acetone. The eluate was evaporated to a constant weight and the residue was dissolved in 1.0 ml DMSO. The DMSO urine concentrates were tested for mutagenicity in the reverse and forward mutation assays, as described further. Dilutions of concentrates were performed until spontaneous background mutation figures were attained. Dilutions of up to 1:1600 were in steps of 2-fold dilution, beginning with a 1:1 dilution. Both strains were examined in the presence and absence of rat liver S9 fraction prepared by methods reported elsewhere (34).

The *Salmonella*/microsomal plate incorporation reverse assay was conducted as described by Maron and Ames (35), using *Salmonella typhimurium* strains TA1535 and TA98 (kindly supplied by Dr. B. N. Ames, University of California at Berkeley).

Forward mutagenicity assays were performed using the TM677 strain (donated by Dr. W. G. Thilly of the Massachusetts Institute of Technology), using media and quality assurance described elsewhere (34). For the assay, an aliquot of 1 μl of the DMSO urine concentrate and positive and negative controls were added, as appropriate, to 99 μl of the reaction medium (34). After incubation in the dark for 2 h, plating was then performed (34).

Statistical Treatment

All data were normalized to CR and a 24-h basis. Typically, the mass or mutagens in the volume tested were multiplied by the inverse of the volume fraction tested to obtain the total in the original urine sample, the CR level was estimated similarly, and the CR-normalized measure was then calculated.

Regression comparisons (36) were performed on the amount of CP, ADR, 5-FU, MTX, and VIN. MEG and PRO were each given to one patient only and so were not considered. Three was the minimum number of data pairs analyzed relative to GA, THIO, mutagenesis, and elements, uncorrected and corrected for personal baseline. The data analysis was subdivided into comparisons of CP with and without ADR and combined for all measures. An aim of the study was to assess whether personal baseline data were necessary for interpretation of the biological monitoring results and for comparison with results of a typical epidemiological study where the dosages were not known. Tests of normality of data were performed using the χ^2 test (36). One- and two-way analyses of variance were done (36).

RESULTS

Subjects

Table 1 gives the questionnaire information obtained from the controls and cancer patients regarding age, gender, and smoking status. Seventy-three % of the cancer patients were older than 60 years, compared with 20% of the controls. One of the cancer patients and four of the controls were men. Smoking status was similar for both groups (about 80% nonsmokers).

Table 2 gives the medications taken by each subject, as well as personal data. The range of i.v. administered CP per patient was 600–1200 mg (2.87–4.60 mmol). This was generally lower when ADR was also given. The range of doses for ADR administered i.v. was 60–80 mg (0.110–0.147 mmol) per patient (patients 7–10). No subjects receiving any one neoplastic drug alone could be recruited. Medications such as steroids, diuretics, antiemetics, sedatives, and analgesics were also taken prior to or concurrently with chemotherapy. Only CP was administered to all cancer patients.

Other antineoplastic drugs were administered to several of the cancer patients. Patients 2, 3, and 5 also received MTX; patients 3, 5, B, and 7 were administered 5-FU; patient A, PRO; patients 1, 4, and 6, VIN; and patient 2, MEG. Only MTX and 5-FU were administered at levels comparable to CP. In contrast, 5 of 15 control subjects took medications (diuretics, antihypertensives, and sedatives).

For one of the cancer patients (patient 7), the urine samples

Table 1 *Distribution of cancer patient and control subject characteristics*

Characteristic	Frequency of characteristic (%)	
	Controls	Cancer patients
Age (years)		
<40	9/15 (60)	1/11 (9)
40–60	3/15 (20)	2/11 (18)
>60	3/15 (20)	8/11 (73)
Smoking status		
Nonsmoker	12/15 (80)	9/11 (82)
Smoker, less than ½ pack daily	1/15 (7)	2/11 (18)
Smoker, more than ½ pack daily	2/15 (13)	0/11 (0)
Gender		
Male	4/15 (27)	1/11 (9)
Female	11/15 (73)	10/11 (91)

Table 2 *Medications taken by cancer patients and control subjects*

Patient	Age (years)	Drug[a]	Dosage[b]
Cancer patients			
A[c]	79	Cyclophosphamide	1000 mg i.v.
		Procarbazine	30 mg p.o. qid
		Prednisone	20 mg p.o. tid
		Phenytoin[d]	100 mg p.o. bid
		Digoxin[d]	0.25 p.o. qd
		Furosemide[d]	40 mg p.o. qd
		Allopurinol[d]	300 mg p.o. qd
1[e]	63	Cyclophosphamide	1200 mg i.v.
		Vincristine	2 mg i.v.
		Prochlorperazine	10 mg i.v.
2	65	Cyclophosphamide	890 mg i.v.
		Methotrexate	40 mg i.v.
		Megestrol acetate	40 mg p.o. qid
		Dexamethasone sodium phosphate[d]	8 mg p.o. qd
		Chlorpromazine	25 mg p.o. 6, 12 h after CP
		Acetaminophen	325 mg p.o. bid
		Quinine sulfate[d]	325 mg p.o. bid
		Hydrochlorothiazide[d]	50 mg p.o. qd
		Oxycodone and acetaminophen	85 mg p.o., 1 Tab
3[f, g]	32	Cyclophosphamide	1080 mg i.v.
		Methotrexate	1080 mg i.v.
		5-Fluorouracil	1080 mg i.v.
		Levothyroxine sodium[d]	0.2 mg p.o., 1 Tab
		Heloperidol	2 mg p.o. tid
		Dexamethasone sodium phosphate	8 mg p.o. tid
4	45	Cyclophosphamide	800 mg i.v.
		Prochlorperazine	10 mg i.v.
		Vincristine	1.0 mg i.v.
		Dexamethasone sodium phosphate	5 mg p.o. qd
5[e, g]	50	Cyclophosphamide	900 mg i.v.
		Methotrexate	70 mg i.v.
		5-Fluorouracil	900 mg i.v.
		Levothyroxine sodium	0.2 mg p.o. qd
		Verapamil hydrochloride	80 mg gu tid
B	70	Cyclophosphamide	800 mg i.v.
		Adriamycin	80 mg i.v.
		5-Fluorouracil	800 mg i.v.
		Lorazepam	2 mg p.o. tid
		Hydromorphone hydrochloride	2 mg p.o. prn
		Dexamethasone sodium phosphate	8 mg p.o. qd
		Chlorpromazine	25 mg p.o. qd
C and 6	77	Cyclophosphamide	750 mg i.v.
		Adriamycin	60 mg i.v.
		Vincristine	2 mg i.v.
7	89	Cyclophosphamide	750 mg i.v.
		Adriamycin	75 mg i.v.
		5-Fluorouracil	750 mg i.v.
		Prochlorperazine	10 mg i.v.
8[f]	70	Cyclophosphamide	600 mg i.v.
		Adriamycin	60 mg i.v.
		Prochlorperazine	10 mg i.v.
		Betamethasone sodium phosphate	6 mg
		Dexamethasone sodium phosphate	5 mg p.o. qd

Table 2 *Continued*

Patient	Age (years)	Drug[a]	Dosage[b]
Control subjects			
1[c]	35	None	
2[c, e]	50	None	
3[e]	37	Triamterene and hydrochlorothiazine	1 Cap p.o. qd
4[c]	71	Furosemide	40 mg p.o. qd
		Potassium bicarbonate	1 Tab p.o. qd
		Citrate quinidine	1 Tab p.o. qd
5	72	Triamterene and hydrochlorothiazide	1 Cap p.o. qd
6	34	None	
7	34	None	
8	32	None	
9[c]	35	None	
10	68	Triamterene and hydrochlorothiazide	1 Cap p.o. qd
		Isosorbide dinitrate	5 mg p.o. bid
		Methyldopa	500 mg p.o. bid
11	41	None	
12	35	None	
13	58	Ibuprofen	400 mg p.o. bid
		Clorazepate dipotassium	3.75 mg p.o. bid
		Amiloride HCl-hydrochlorothiazide	1 Tab p.o. qd
		Thiazide	
14	33	None	
15[e]	36	None	

[a] Adriamycin, cyclophosphamide, 5-fluorouracil, megestrol acetate, methotrexate, procarbazine, and vincristine are antineoplastic drugs (only ADR, CP, and procarbazine are known mutagens and animal carcinogens). Betamethazone sodium phosphate, dexamethasone sodium phosphate, levothyroxine sodium, and prednisone are steroids; allopurinol is for arthritis; amiloride HCl-hydrochlorothiazide and triamterene and hydrochlorothiazide are diuretic/antihypertensives; digoxin, furosemide, triamterene, and hydrochlorothiazine are diuretics; hydrochlorothiazide and methyldopa are antihypertensives; chlorpromazine and prochlorperazine are antiemetics; acetaminophen, ibuprofen, lorazepam, and oxycodone are analgesics; phenytoin is a cardiac glycoside; quinine sulfate is for muscle cramps; levothyroxine sodium is a thyroid medication; clorazepate dipotassium, haloperidol, and hydromorphone hydrochloride are tranquilizers; verapamil hydrochloride is for eyedrops; potassium bicarbonate is a potassium source; citrate quinidine is a cardiac antiarhythmic; isosorbide dinitrate is a muscle relaxant.

[b] Dosage abbreviations: Cap, capsule; c, chemotherapy; gu, eyedrops; qd, once a day; bid, twice a day; tid, 3 times a day; qid, 4 times a day; prn, take as needed; Tab, tablet.

[c] Male.

[d] Administered prior to chemotherapy.

[e] Smoker: patients 2 and 6 and control 3 smoked <1 pack/day; controls 2 and 15 smoked <0.5 pack/day.

[f] Previous exposure to CP/ADR; patient 2, 1 week; patient 3, 4 weeks; patients 4 and 10, 2 weeks. The rest provided no information but had received previous treatment.

[g] Previous radiation treatment: patient 4, 4 months; patient 6, 5 months; patient 10, none; the rest provided no information.

were obtained after the first chemotherapy treatment. The others had previously received prior treatments, generally at 2 weeks to 1 month (Table 2). Only 1 of the 15 controls did not produce a 24-h urine sample (23 h instead), as did two of the cancer patients (subject 6 did not collect the first void; subject 7 experienced occasional incontinence following CP/ADR treatment). Three of the cancer patients did not collect the pretreatment sample.

Chemical Parameters

Creatinine/Urine Volumes. The CR results, 24-h urine volumes, and treatment dosages are summarized for the controls and cancer patients (pre- and posttreatment) in Tables 3 and 4, respectively, along with the THIO and GA data. For the CR concentrations (Fig. 1), none of the data were distributed normally; therefore, arithmetic means and SD values are not representative. The data for the controls followed a bimodal distribution (modes are 4–5 and 11–12 mM). This was not observed for the patients. The lower control mode describes more closely the data from the patients.

There were no marked differences between control and patient pretreatment data. Lauwerys (20) does not recommend normalization to CR for CR <2.65 mM. Urine of 2 of the 11 posttreatment cancer patients [patients 2 (CP/MTX/MEG) and 4 (CP/VIN)], none of the pretreatment cancer patients, and none of the control subjects had CR concentrations <2.65 mM

Table 3 *Urine volumes, creatinine, urinary D-glucaric acid, and thioether data for control subjects*

Subject	Urine volume (ml)	Creatinine		D-Glucaric acid				Thioethers as —SH				Ratio μmol —SH / μmol GA
		Concentration (mM)	Amount (mmol/day)	n[a]	Concentration (μM)	CV (%)[b]	Normalized concentration (μmol/g CR)	Amount (μmol/day)	n	Concentration (μM)	CV (%)	Normalized concentration (μmol/g CR)	Amount (μmol/day)	
1	1445	11.6	17.3	41	35.8 ± 0.6[c]	1.7	27.4	53.5	6	47.2 ± 3.63	7.7	4.08	70.6	0.758
2	978	12.7	12.4	8	44.2 ± 3.2	7.2	30.8	43.2	3	60.9 ± 8.66	14.2	4.80	59.6	0.726
3	[d]	11.1	[d]	4	42.5 ± 2.0	4.7	33.9	[d]	3	62.4 ± 3.52	5.6	5.63	[d]	0.681
4	1335	7.16	9.56	2	33.1 ± 0.9	2.7	40.9	44.2	3	29.0 ± 4.13	14.2	4.05	38.7	1.14
5	685	11.0	7.54	2	25.1 ± 2.4	9.6	20.2	17.2	4	59.8 ± 4.51	7.5	5.44	41.0	0.420
6	2020	4.52	9.13	7	18.0 ± 1.2	6.7	35.2	36.4	5	17.5 ± 1.72	9.8	3.88	35.4	1.03
7	1440	6.81	9.81	4	15.8 ± 1.5	9.5	20.5	22.8	3	25.1 ± 1.10	4.4	3.69	36.1	0.629
8	600	18.9	11.1	5	51.4 ± 0.6	1.2	24.6	30.8	4	70.4 ± 5.1	7.2	3.81	42.2	0.730
9	1490	10.4	15.5	2	25.4 ± 1.4	5.5	21.6	37.8	4	57.5 ± 1.98	3.4	5.53	85.7	0.442
10	1380	4.35	6.00	3	18.0 ± 1.3	7.2	36.6	24.8	2	23.8 ± 1.97	8.3	5.46	32.8	0.756
11	1720	4.52	7.77	6	13.7 ± 1.4	10.2	26.7	23.6	3	18.6 ± 1.22	6.6	4.12	32.0	0.737
12	1500	3.55	5.32	2	16.9 ± 1.6	9.5	42.0	25.4	4	11.5 ± 1.19	10.3	3.25	17.2	1.47
13	1220	5.83	7.11	2	24.8 ± 1.5	6.0	37.6	30.3	4	11.5 ± 1.19	10.3	3.25	17.2	1.47
14	1075	6.63	7.13	2	26.8 ± 2.5	9.3	35.7	28.8	4	27.0 ± 2.52	9.3	4.07	29.0	0.993
15	760	3.98	3.02	2	18.9 ± 0.3	1.6	42.0	14.4	3	30.2 ± 2.82	9.3	7.58	23.0	0.626
Mean ± SD	1264 ± 406	8.20 ± 4.31	9.19 ± 3.89		27.4 ± 11.6	6.2 ± 3.2	31.7 ± 7.76	30.9 ± 11.0		37.9 ± 19.5	8.4 ± 3.1	4.68 ± 1.11	41.2 ± 18.6	0.802 ± 0.271
CV (%)	32.1	52.5	42.3		42.4	52	24.5	35.5		51.5	37	23.6	45.2	33.8

[a] *n*, number of times the sample was assayed.
[b] CV, coefficient of variation.
[c] Mean ± SE.
[d] Not a 24-h sample.

(Fig. 1). All but one control (control 8, 18.9 mM) excreted between 3 and 13 mM CR. Pretreatment levels in cancer patients were between 2 and 18 mM, with 2 between 15 and 18 mM (patients 5 and 7). For posttreatment cancer patients, all were between 1 and 9 mM, with 1 [patient 7 (CP/ADR/5-FU)] at 11.1 mM. There were no obvious effects of gender or age on creatinine or urine volume.

The same data were analyzed in terms of daily excretion of CR. The normal excretion range is 6–13 mmol/day. Two (controls 12 and 15) of the 15 controls and 3 [patients 1 (CP/VIN), B (CP/ADR/5-FU), and C (CP/ADR/VIN)] of the posttreatment cancer patients excreted <6 mmol/day. One (control 1) of the controls and none of the posttreatment cancer patients excreted more than 16 mmoles/day.

The normal range of 24-h urine volumes is 600–2500 ml (19). The control population data were normally distributed and within the normal range. Three of the cancer patients [patients 1 (CP/VIN), 2 (CP/VIN), and 5 (CP/MTX/5-FU)] had 24-h urine volumes >2500 ml. Although patient A and controls 3–5, 10, and 13 were taking diuretics, none produced above normal urine volumes. The interindividual variation was 32% for controls and 56% for treated patients.

D-Glucaric Acid. It was determined that the method of Colombi *et al.* (23) for GA could be reproduced (*i.e.*, experimental calibration curve of $y = 45.4x \pm 6.79$ *versus* estimated curve for Colombi's paper of $y = 50.6x \pm 9.53$, where y is absorbance units and x is mmol GA). Reference urine from a male control subject was analyzed along with every group of samples for quality control. The initial analysis resulted in an arithmetic mean and 1 SD of 26.6 ± 0.2 μmol GA/g CR. Colombi *et al.* (23) reported an interrun coefficient of variation of 7–9% and an intrarun coefficient of variation of 4–6%. The reference urine over the 12-month period of the study was characterized by 12% ($n = 17$) and 8% ($n = 8$) for these respective parameters.

Attempts to improve Colombi's method by varying the pH and concentration of the glycine buffer, substrate and enzyme concentrations, incubation time and temperature, and substrate type [there was no advantage to 4-nitrophenyl-β-glucuronide, as used by Jung *et al.* (37)] did not lead to better sensitivity, although the values of the controls varied. There were no differences in the coefficients of variation between GA expressed as a concentration, daily amount excreted, or creatinine-adjusted concentration for cancer patients (Table 4). For controls (Table 3) the creatinine-adjusted concentration showed less interindividual variation than the other two parameters (the range was 20–50 μmol GA/g CR). The precision of measurement on each sample (12–19%) accounted for around half of the interindividual variation (25–59%). Fig. 2 shows the creatinine-adjusted GA data for the controls and pre- and posttreatment cancer patients. The control values ranged between 20 and 45 μmol/g CR for preexposure patients, only two [patients 5 (CP/MTX/5-FU) and 7 (CP/ADR/5-FU)] were above 45 μmol/g CR, whereas of the posttreatment patients 82% produced urine above this level. Thus, administration of antineoplastic drugs increased GA excretion. It is also clear that the data are not normally distributed.

Six of the treated cancer patients [patients A (CP/PRO/VIN), 2 (CP/MTX/MEG), 4 (CP/VIN), 5 (CP/MTX/5-FU), 7 (CP/ADR/5-FU), and 8 (CP/ADR)] had 24-h GA excretion greater than the highest observed for the control subjects, namely, 53.5 μmol/day. Patients 1 (CP/VIN), 3 (CP/MTX/5-FU), B (CP/ADR/5-FU), and C (CP/ADR/5-FU) produced urine with GA concentrations in the range of control urine. There is clearly much individual variation even in people treated

Table 4 *ADR/CP dose, urine volumes, creatinine, D-glucaric acid, and thioether data for cancer patients*

Patient	Treatment: Pre/post	Treatment: Drug (mg)	Urine volume (ml)	Creatinine: Concentration (mM)	Creatinine: Amount (mmol/day)	D-Glucaric acid: n[a]	D-Glucaric acid: Concentration (μM)	D-Glucaric acid: CV (%)[b]	D-Glucaric acid: Normalized concentration (μmol/g CR)	D-Glucaric acid: Amount (μmol/day)	Thioethers as —SH: n	Thioethers as —SH: Concentration (μM)	Thioethers as —SH: CV (%)	Thioethers as —SH: Normalized concentration (μmol/g CR)	Thioethers as —SH: Amount (μmol/day)	Ratio μmol —SH / μmol GA
A	Post	CP (1000)	1160	5.14	5.96	2	305 ± 27[c]	8.9	526	354	5	45.0 ± 7.7	7.5	8.85	52.8	0.149
1	Pre			2.65		3	7.5 ± 1.2	16.2	24.9		2	15.0 ± 5.2	24.6	5.66		2.01
	Post	CP (1200)	2620	3.09	8.10	4	15.9 ± 1.6	10.1	45.4	41.7	3	15.2 ± 4.6	17.6	4.92	39.8	0.956
2	Pre			7.21		3	35.0 ± 1.5	4.3	42.9		5	26.5 ± 4.5	7.5	3.68		0.757
	Post	CP (890)	3920	1.89	7.29	4	19.1 ± 3.7	19.4	91.0	79.9	4	9.75 ± 2.05	10.5	5.24	38.2	0.510
3	Pre			7.16		3	22.7 ± 2.1	9.4	28.0		3	32.1 ± 4.2	7.5	4.48		1.41
	Post	CP (1080)	1130	8.17	9.23	4	47.1 ± 1.8	3.8	51.0	53.2	4	85.3 ± 10.6	6.2	10.4	96.4	1.81
4	Pre			3.71		3	29.1 ± 1.6	5.5	69.3		3	21.7 ± 3.7	9.9	5.86		0.746
	Post	CP (800)	2365	2.55	6.03	4	40.1 ± 5.0	12.5	139	94.8	3	39.8 ± 5.9	8.5	15.6	94.1	0.993
5	Pre			17.2		3	74.2 ± 5.5	7.4	38.1		3	182 ± 12.	3.8	10.6	10.6	2.45
	Post	CP (900)	2750	3.18	8.74	4	28.5 ± 3.3	11.6	79.2	78.4	3	44.7 ± 2.9	3.7	14.1	123	1.57
B	Post	CP (800) ADR (80)	698	6.29	4.39	5	29.5 ± 1.0	3.4	41.4	20.6	3	83.5 ± 5.7	3.9	13.3	58.5	2.83
C	Post	CP (750) ADR (60)	750	5.19	3.89	2	73.2 ± 0.1	0.1	125	54.5	4	97.8 ± 19.7	10.1	18.3	73.3	1.34
6	Pre			10.6		4	33.8 ± 2.4	7.1	28.2		2	34.6 ± 2.7	5.4	3.26		1.02
	Post	CP (750) ADR (60)	940[d]	7.89	[d]	4	67.0 ± 5.6	8.4	75.3	[d]	3	91.2 ± 15.7	9.9	11.6	[d]	1.36
7	Pre			15.1		3	59.3 ± 3.8	6.4	34.7		4	70.8 ± 6.6	4.7	4.68		1.19
	Post	CP (600)		11.1	[d]	3	67.6 ± 6.2	9.2	53.8	[d]	5	48.7 ± 4.6	4.2	4.39	[d]	0.721
8	Pre			4.08		3	48.0 ± 2.6	5.4	104		4	53.2 ± 14.7	13.8	13.0		1.11
	Post	CP (600)	2365	3.24	7.66	4	45.4 ± 6.4	14.1	124	107	6	35.1 ± 6.5	7.5	10.8	83.0	0.773
Mean ± SD[e]			1973 ± 1096	6.53 ± 4.52	6.25 ± 2.16		43.3 ± 20.6[f]		82.5 ± 36.1[f]	66.3 ± 28.8[f]		55.1 ± 32.1[g]		10.7 ± 4.64	73.2 ± 28.6[f]	1.29 ± 0.677[f]
CV (%)			55.6	69.2	34.5		47.6		43.8	43.4		58.2		43.4	39	52.5
Mean ± SD[h]				6.53 ± 4.52			38.7 ± 21.2		46.3 ± 27.2			36.3 ± 19.4[g]		16.6 ± 9.99		1.34 ± 0.604
CV (%)				69.2			54.7		58.8			53.5		60.2		45.1

[a] *n*, number of times the sample was assayed.
[b] CV, coefficient of variation.
[c] Mean ± SE.
[d] Not a 24-h sample.
[e] For all posttreatment samples.
[f] Statistics (mean, SD, and CV) are without subject A.
[g] Statistics (mean, SD, and CV) are without subject 5.
[h] For all pretreatment samples.

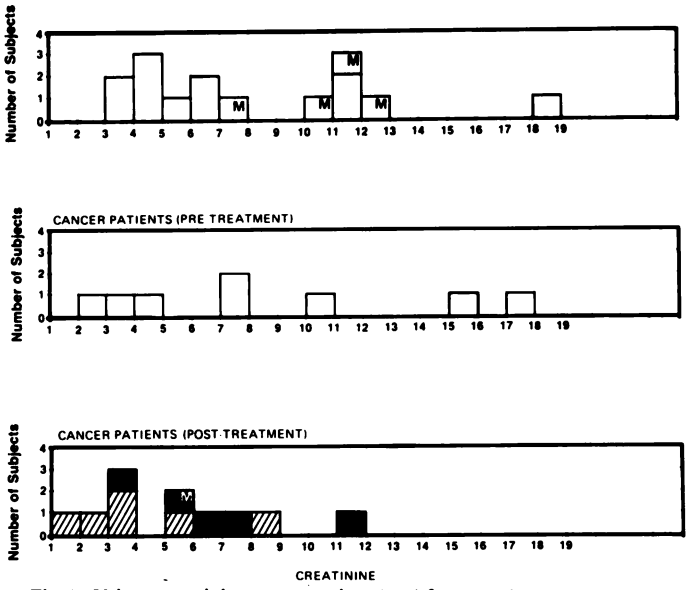

Fig. 1. Urinary creatinine concentrations (mM) for control subjects and cancer patients. *M*, samples from a male subject. ▨, cyclophosphamide treatment; ■, cyclophosphamide/adriamycin combined treatment.

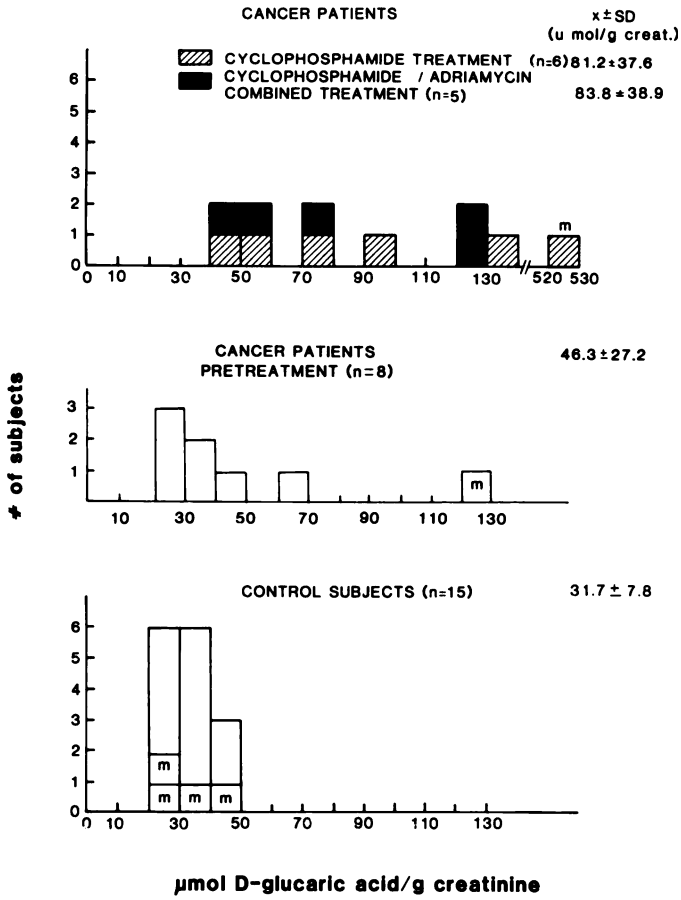

Fig. 2. Creatinine-adjusted D-glucaric acid concentrations for control subjects and cancer patients.

with the same regimens. The data are normally distributed for both groups, if patient A is excluded, and range from 14.4 to 53.5 μmol/day for the controls and 20.6 to 94.8 μmol/day for the treated cancer patients. The group arithmetic means for the control subjects (30.9 ± 11.0 μmol/day) and treated cancer patients, excluding patient A (66.3 ± 28.8 μmol/day), show a statistically significant difference in 24-h GA excretion by the treated cancer patients ($t = 3.34$, $df = 21$, $P < 0.01$). CP in the absence of ADR treatment produces the same results as the CP/ADR regimen ($t = 0.33$, $df = 7$, $P > 0.05$).

Another way of analyzing the data is to use the preexposure sample as the person's own control. Fig. 3 compares the creatinine-adjusted GA levels for each subject for whom pretreatment and posttreatment samples were collected. In all cases, the posttreatment value is statistically significantly different from the pretreatment sample. The signed range test [a nonparametric statistical test for paired samples (38)] indicated that the means of the pre- *versus* posttreatment groups were significantly different ($P < 0.01$). Except for patient 8, all pretreatment values for patients were no different from values included in the range for the controls.

Thioethers. The molar absorptivity for —SH determination using the Ellman reagent with reduced glutathione as substrate was 13.6 ± 2.5 liter mol^{-1} cm^{-1}, as compared with Ellman's reported value of 13.6 liter mol^{-1} cm^{-1} (39). The data for the reference urine over 12 months showed an intrarun coefficient of variation of 13% and an interrun coefficient of variation of 8%. Use of methyl acetate (only 75–83% efficient), different methods of shaking, and varying the amount of excess sodium chloride (>1 g caused a colloidal solution that could not be processed) and number of extractions (15 min of shaking with 3 ml urine for two extractions led to no measurable —SH levels above control values after the second extraction) did not enhance the sensitivity of the technique. Van Doorn *et al.* (27) have recommended the use of a normal upper concentration of 5.9 mmol SH/mol CR.

Tables 3 and 4 contain the THIO data for the controls and cancer patients. Fig. 4 depicts the CR-adjusted THIO levels for the control subjects and pre- and posttreatment cancer patients. None of the THIO data except for the controls are normally or lognormally distributed. The controls had THIO levels between 3.25 and 7.58 (arithmetic mean ± SD, 4.68 ± 1.10; median, 4.78) mmol SH/mol CR, with nearly 73% of the values between 3.5 and 5.5 and one above 6. For patients before exposure, two (patients 5 and 8) had values above 6 mmol SH/mol CR, whereas 75% of the exposed patients had levels above this value. Patients 1 (CP/VIN), 2 (CP/MTX/MEG), and 7 (CP/ADR/5-FU) produced urine with levels below this value. Cancer patients treated with CP but with no ADR had a group arithmetic mean of 9.85 ± 4.43 (median, 9.62) mmol SH/mol CR. Cancer patients treated with CP/ADR had a group mean of 11.8 ± 5.18 (median, 11.6) mmol SH/mol CR. The Wilcoxin rank sum test showed a statistically significant difference ($P < 0.01$) in the group medians for both the CP without ADR-

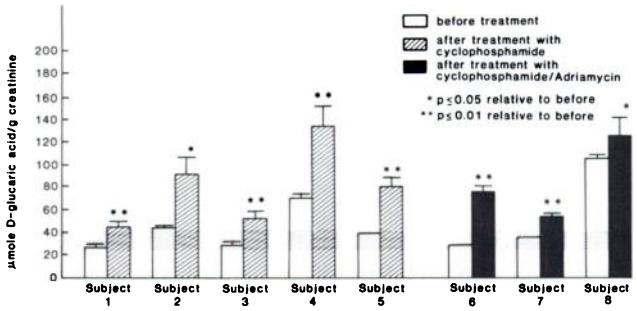

Fig. 3. Creatinine-adjusted D-glucaric acid concentrations in individual cancer patients before and after treatment with antineoplastic drugs. Horizontal shaded area indicates ± SD from the mean of 15 control subjects.

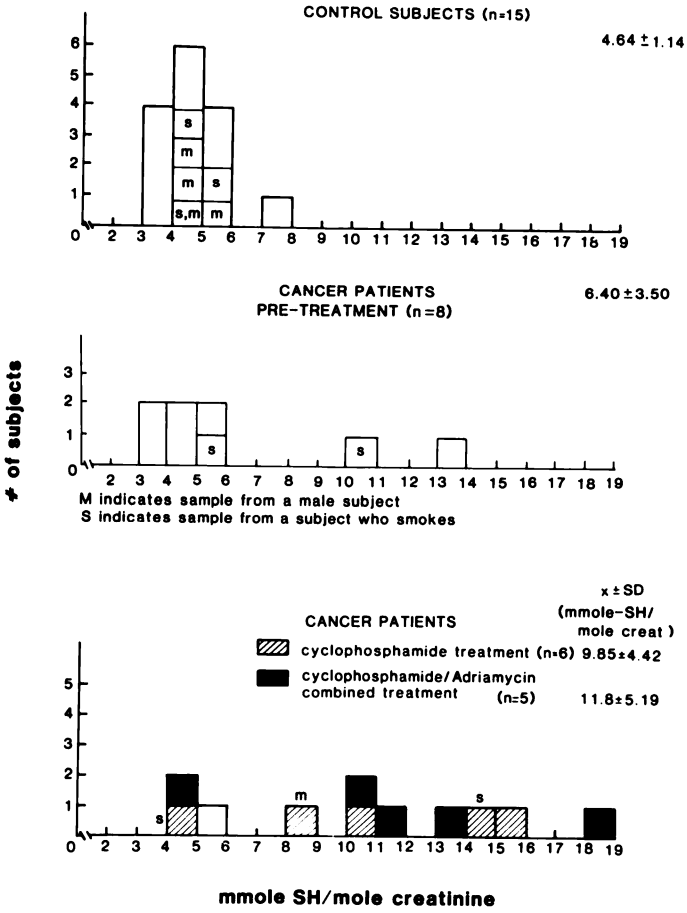

Fig. 4. Creatinine-adjusted thioether concentrations for control subjects and cancer patients.

treated and CP/ADR-treated cancer patients, compared to the control subjects. No statistically significant difference ($P > 0.05$) was found between the CP without ADR- and CP/ADR-treated group arithmetic means.

Fig. 5 compares the CR-adjusted THIO levels for each subject for whom pretreatment and posttreatment samples were available. Statistically significant ($P < 0.01$ or 0.05) differences in the pre- *versus* posttreatment values were seen in five of the eight subjects (Fig. 5). One of these, patient 8, had higher pre- and posttreatment THIO concentrations than the controls. The nonparametric signed rank test (38) showed no statistically significant difference between the means of the pre- *versus* post-treatment groups ($P > 0.05$).

The values for THIO excreted in controls in 24 h ranged from 17 to 86 μmol SH/day, with 60% of the values between 30 and 60. After exposure, 60% of the patients had levels above 60 μmol SH/day. Patients A (CP/PRO/VIN), 1 (CP/VIN), 2 (CP/MTX/5-FU), and B (CP/ADR/5-FU) produced urine with levels below this value. The data for the controls and exposed cancer patients are not normally distributed. A logarithmic transformation of the data achieves normality, as shown by the Shapiro Wilks test (38). The group geometric means are statistically significantly higher for the post-CP treatment cancer patients, compared to the control subjects ($t = 2.46$, $df = 19$, $0.01 < P < 0.05$). The combined CP without ADR and CP/ADR treatment groups were also statistically significantly different from the control subjects ($t = 3.27$, $df = 22$, $P < 0.01$; and $t = 4.10$, $df = 15$, $P < 0.01$, respectively).

The coefficient of variation is much more precise for CR-normalized concentrations, for cancer patients, than for daily amounts excreted. Around 70% of the SD for normalized concentrations is caused by the coefficient of variation for individual sample precision (16). There were no such differences for controls.

Elements. Table 5 shows the elemental content of the urine from cancer patients after antineoplastic drug treatment, compared with the urine of 10 of 15 controls. No pretreatment urine was available for personal baseline analysis for the patients, because it was decided to use all of these samples for mutagenicity testing because of the limited volume. Since the urine from controls 1–3 and 15 also were of limited volume, the same decision was made there also.

The elements significantly altered in patients relative to controls were C (increase in 7 of 10), S (increase in 7 of 10; decrease in 1 of 10), P (increase in 5 of 10); decrease in 2 of 10), and Na (increase in 4 of 10). The CP/VIN combination appeared to cause increases in Na, P, and S. The CP/ADR/5-FU combination (patients B and 7) only caused a consistent increase in C. All other combinations showed variable results.

The results for phosphorus are of interest since CP contains phosphorus. Patients 1 and 4 (CP/VIN), 5 (CP/MTX/5-FU), and 6 (CP/ADR/5-FU) showed increases; patients 3 (CP/MTX/5-FU) and 7 (CP/ADR/5-FU) showed decreases relative to the range exhibited by control urine. The rest of the urine samples of the patients showed concentrations in the same range as control urines. If all the results for each element are considered, only K, P, and S showed significant increases above control urine samples at $P \leq 0.05$ (38).

Mutagenicity. The results of testing ADR and CP themselves are reported in detail elsewhere (34). In summary, ADR was not mutagenic in the reverse assay to strain TA1535 at up to 10 μg/plate; at 100 μg/plate it was toxic, a phenomenon also noted for strain TA98. ADR was mutagenic without the need for added rat S9 fraction. The lower limit for ADR mutagenicity in strain TM677 in the forward assay was about 0.5 μg ADR/ml, with a viability of only 60% (at ≤0.10 μg/ml, viability was 100%). CP itself was shown to be mutagenic in a non-dose-dependent manner only in strain TA1535 in the reverse assay, with no toxicity observed at up to 400 μg/plate. Similarly, TM677 did not detect CP mutagenicity in the forward assay. A detailed description of these experiments can be found elsewhere (34).

Urine from patients treated with CP/ADR gave the results shown in Table 6*A*. The units are given in total revertants for the entire urine sample or are normalized to CR. The relative errors were typically of the order of 5 to 10%. The values selected from the 100-ml urine aliquots had to have at least

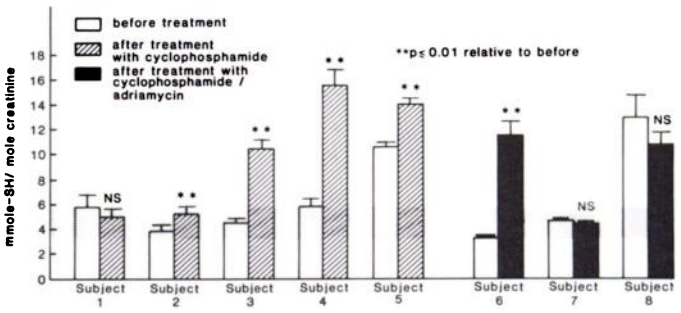

Fig. 5. Creatinine-adjusted thioether concentrations in individual cancer patients before and after treatment. Horizontal shaded area indicates ± SD from the mean of 15 control subjects.

Table 5 *Elemental composition of urine of cancer patients and controls*

Subject	Concentrations (mg element/mmol creatinine)														
	Ca	Cu	Fe	I	K	Li	Mg	Na	Ni	P	S	Si	Sr	Zn	C
Patient															
A	2.55[a]	<0.006	0.0136	117[b]	139	<0.002	3.67	133	<0.02	101	46.5[a]	0.349	0.0123	<0.05	639
1	20.3	0.017[b]	<0.01	<16	307	<0.003	8.17	615[b]	0.090[b]	128[b]	164[b]	0.714	0.0317[b]	0.0942	1,618[b]
2	0.656[a]	<0.016	<0.027	<26	248	<0.005	2.79	745[b]	<0.05	106	124[b]	<0.53	<0.01[a]	0.176	14,931
3	11.4	<0.004	<0.006	<6	208	<0.001	2.97	419	<0.01	44.1[a]	95.4	0.327	0.0110	<0.03	792
4	15.9	<0.01	<0.02	<20	598[b]	<0.004	12.7	1,235[b]	<0.04	163[b]	197[b]	0.585	0.0188	0.128	1,905[b]
5	9.48	<0.009	<0.02	<16	231	<0.003	5.30	282	<0.03	133[b]	125[b]	<0.3	0.0170	0.125	956
B	5.13	0.0130[b]	0.0086	<8	183	0.0041[b]	5.55	223	<0.02	106	71.5	0.169	0.0121	0.387	1,261[b]
6	9.98	<0.006	0.0143	<10	416[b]	0.0019[b]	10.2	643[b]	<0.02	175[b]	195[b]	0.375	0.0146	0.0983	2,042[b]
7	8.15	<0.003	<0.005	<5	186	<0.009	5.01	298	<0.009	53.1[a]	122[b]	0.667	0.0197	0.0788	1,249[b]
8	11.5	<0.009	0.0188	<15	236	<0.003	8.98	811[b]	<0.03	92.7	120[b]	1.15	0.0194	0.182	6,623[b]
Arithmetic mean ± SD	9.50 ± 5.90	0.015 ± 0.003	0.0138 ± 0.0042	117	275 ± 136[b]	0.0030 ± 0.0016	6.53 ± 3.33	520 ± 363	0.090	110 ± 42	126 ± 49[c]	0.542 ± 0.309	0.0174 ± 0.0063	0.159 ± 0.099	3,202 ± 4,470
Mode (%)[d]	8–10 (30)				160–200 210–260 (30)		5–6 (40)	130–230 230–330 530–630 730–830 (18)		100–110 (30)	120–130 (40)	0.3–0.4 (38)	0.018–0.020 (33)	0.08–0.10 0.12–0.14 (25)	1,200–1,300 (20)
Control															
4	6.57	<0.004	<0.07	<7	366	0.0014	12.9	360	<0.01	116	83.8	0.889	0.0088	0.0712	1,021
5	6.67	<0.003	0.0049	<5	196	0.0009	7.99	215	<0.01	94.5	85.4	<0.10	0.0075	0.140	815
6	7.45	<0.007	<0.01	<11	189	<0.002	7.45	301	<0.02	80.4	90.0	0.290	0.0142	<0.06	790
7	14.5	<0.004	<0.007	<8	69.4	<0.002	9.90	247	<0.01	61.4	52.1	0.177	0.0186	<0.04	525
8	9.80	<0.002	0.053	<3	205	<0.005	8.44	173	<0.005	88.5	66.2	0.321	0.0083	0.0220	827
9	10.4	<0.003	0.0174	<5	92.2	<0.001	2.34	372	<0.01	83.5	69.3	<0.10	0.0101	<0.02	450
10	5.14	<0.007	<0.01	<11	320	<0.002	10.4	546	<0.02	105	120	0.882	0.0136	<0.06	1,071
11	14.5	<0.007	<0.01	<11	150	<0.002	8.19	263	<0.02	71.2	91.7	0.460	0.0133	<0.06	725
12	16.4	<0.009	0.0532	<14	210	<0.003	11.8	357	<0.03	87.9	93.5	1.16	0.0146	<0.07	973
13	23.5	<0.005	<0.009	<9	122	<0.002	7.06	452	<0.02	86.3	95.3	0.463	0.0232	<0.04	875
14	12.3	<0.005	<0.008	<8	134	<0.002	5.21	354	<0.02	68.4	71.9	0.217	0.0161	0.0388	656
Arithmetic mean ± SD	11.6 ± 5.4	<0.009	0.0353 ± 0.0253	<14	141 ± 101	0.0012 ± 0.0004	8.33 ± 2.96	331 ± 108	0.02	76.6 ± 27.8	85.8 ± 17.4	0.540 ± 0.350	0.0135 ± 0.0047	0.0680 ± 0.0522	793 ± 196
Mode (%)	6–8 (27)				160–210 (36)		7–8 (27)	330–430 (36)		80–90 (45)	90–100 (56)	0.2–0.3 0.4–0.5 0.8–0.9 (22)	0.008–0.010 0.012–0.014 (18)	0.02–0.04 (50)	800–900 (27)

[a] Lower than lowest value in controls.
[b] Higher than highest value in controls.
[c] Different from control by $P \leq 0.05$ (two-tailed test) (38).
[d] Mode (%) signifies the mode range and percentage values of the total falling in that range for data with 50% of values above the least quantifiable level.

Table 6 *Mutagenicity results for the cancer patients and control subjects*

	Reverse assay						Forward assay TM 677			
	TA 1535				TA 98		A		B	
	A[a]		B[b]		A	B				
Patient	−S9	+S9	−S9	+S9	−S9	−S9	−S9	+S9	−S9	+S9
A	35,400	36,000	5,940	6,030	BD[c]	BD	916	1,020	154	171
1	12,400	139,000	15,400	17,100	BD	BD	812	1,074	100	132
2	73,300	76,000	10,100	10,400	BD	BD	3,610	3,720	495	511
3	54,200	43,600	5,880	4,730	BD	BD	418	723	45.3	78.3
4	49,700	51,300	8,240	8,510	BD	BD	828	4,730	137	784
5	187,000	128,000	21,400	14,700	BD	BD	[d]	[d]	[d]	
B	44,300	48,500	10,080	11,100	4,330	1,400	1,540	[d]	350	[d]
C	48,500	54,500	12,500	14,000	3,490	900	1,500	3,830	386	983
6	96,800	95,700	13,100	12,900	5,260	710	[d]	[d]	[d]	[d]
7	[d]	[d]	11,400	10,300	[d]	1,340	[d]	[d]	[d]	[d]
8	66,700	65,000	8,710	8,490	5,680	740	4,269	3,780	556	494

Strain	Data
Dimethyl sulfoxide blank	
TA 1535	−S9 12 ± 8; +S9, 5 ± 3
TA 98	−S9 9 ± 4
TM 677	−S9 (3.3 ± 1.1) mutants/10^5 survivors, >80% viability
	+S9 (5.4 ± 1.0) mutants/10^5 survivors, >80% viability
Range for subjects not exposed to antineoplastic drugs	
TA 1535	−S9 10–30 revertants/plate; mean ± SD, 17 ± 6 (n = 13)
	+S9 8–38 revertants/plate; mean ± SD, 16 ± 9 (n = 13)
TA 98	−S9 6–30 revertants/plate; mean ± SD, 18 ± 8 (n = 13)
TM 677	−S9 3.1–7.6 mutants/10^5 survivors; mean ± SD, 4.7 ± 2.0 (n = 4)
	+S9 3.7–6.6 mutants/10^5 survivors; mean ± SD, 4.9 ± 1.3 (n = 4)
Positive controls results	
TA 1535	−S9 Positive *N*-methyl-*N'*-nitrosoguanidine spot test, 4+
TA 98	−S9 1-Amino-2-carboxy-4-nitroanthraquinone 150 μg/plate, 2292 ± 115 revertants/plate (3)
TM 677	−S9 ICR-191, 10 μg/ml: 200 ± 32 mutagens/10^5 survivors (3)
	+S9 2-Azaguanine, 10 μg/ml: 510 ± 128 mutants/10^5 survivors (3)

[a] A, total revertants in urine/24 h.
[b] B, revertants/mmol creatinine.
[c] BD, revertants/plate is less than 2 times the dimethyl sulfoxide solvent blank plate.
[d] Sample lost.

twice the number of revertants as in control samples (Table 6*C*). The values which agreed within 10% were then averaged and the target parameters were calculated. Table 6*B* provides the data for the dimethyl sulfoxide solvent blank, as does Table 6*D* for the positive controls.

In the reverse assay, no control subjects showed mutagenicity with strain TA1535 without S9. When S9 fraction was added, the potency was significantly decreased in urine from patients 3 and 5. In the forward assay, addition of S9 increased mutagenicity for patients 1, 3, 4, and C. The potency for control subjects relative to DMSO blanks was increased but added S9 had no effect; however, a decrease was observed for TA1535. Strain TA98 did not detect CP-exposed patients, whereas both TA1535 (>0.1 mmol administered CP/plate) and TM677 did. Strain TA98 detected ADR (>0.1 mmol administered ADR/plate) without the addition of S9. The TM677 test was less sensitive than the TA1535 one, since mutagenicity was detected between dilutions of 1:10 and 1:50 for TM677, compared with 1:100 to 1:1000 for TA1535.

DISCUSSION

Creatinine Assay. The only substance whose concentration has been used to any extent for adjusting urine for dilution variability is CR (19). The clearance rate of CR depends on body muscle mass and on the glomerular filtration rate (40). The cancer patients were older than control subjects, with fewer males, and were generally not as healthy (because of either their disease or the effects of the treatment). Thus, their body muscle mass should be less than that of the control subjects. This might explain the lower 24-h CR excretion found in the cancer patients. The differences, however, were not significant when only the female control subjects were pooled. Also, the patient (subject A) and the four controls (Table 2) who took diuretics did not have abnormal 24-h urine volumes or CR concentrations. Furthermore, it is very difficult to obtain a complete 24-h urine sample, particularly from elderly female patients who are nauseated and have diarrhea (common side effects of antineoplastic drugs). No subject reported missing a specimen collection. Lauwerys (20) has recommended that very dilute urine with CR less than 2.65 mM must not be used for normalization purposes. However, only 2 patients (patients 2 and 4) of the 11 posttreatment cancer patients had very dilute urine, perhaps due to medications (*e.g.*, steroids) or to the fact that the cancer patients were encouraged to drink large volumes of water to minimize the toxic effect CP has on the bladder. Three of the 11 treated cancer patients had 24-h urine volumes greater than the 2500 ml considered average (19). The inability to concentrate urine may indicate tubular dysfunction (40). The pretreatment samples, however, were adequately concentrated (*i.e.*, within the normal range for CR concentration), which does not indicate intrinsic dysfunction.

Adjusting data for the dilution of the urine on the basis of specific gravity or osmolality, as is done by other investigators, is not particularly appropriate in this case, because drugs contribute to changes in specific gravity and osmolality.

D-Glucaric Acid Assay. The GA assay developed by Colombi *et al.* (23) was shown to be rugged and already optimized. GA concentrations for the 15 control subjects were within 2 SD of the group (573 subjects) means reported by Colombi *et al.* (23).

Colombi *et al.* (23) observed a mean of 26.9 ± 8.7 μmol GA/g CR when testing 402 males and 29.7 ± 8.4 for 171 females. In this study, the group mean (control subjects) for the 4 males was 30.2 ± 8.1 μmol GA/g CR, and for the 11 females it was 32.3 ± 8.0. No statistically significant difference was determined between the male and female group means for the control subjects, between the males in this study and the males in Colombi's investigation, or between the females in this study and the females in Colombi's work. Thus, the same normal values for Europeans in the United States were found as for Europeans in Europe.

Thioether Assay. THIO levels for the 15 control subjects were within 2 SD of group means reported by Van Doorn *et al.* (27). Thus, the same normal values as in Europe were confirmed for the Caucasian population studied. Van Doorn *et al.* (27) proposed an upper normal limit of 5.90 mmol SH/mol CR, a value not exceeded in any of the control subjects of the present study.

An increase in THIO levels would be expected in humans exposed to CP, in that a specific THIO (3-hydroxypropylmercapturic acid) has been detected in the urine of rats treated with CP (41). However, urine collected 6 h after CP (1000 mg, i.v.) from five patients showed variable amounts of 3-hydroxypropylmercapturic acid excretion (41). The lowest levels found were 6.4 and 9.9 μmol; one was 17.5 μmol, and the highest values found were 40 and 50 μmol. No correlation between dose administered and 3-hydroxypropylmercapturic acid excretion was apparent. This supports the results of the present study (*i.e.*, no apparent correlation between CP dose and THIO over the CP dosage levels studied). The 24-h THIO was 73.2 ± 28.6 μmol —SH/24 h for the cancer patients treated with CP or CP/ADR in this study.

Elements. ADR is a known metal-chelating agent and effects on metals in tissues have been postulated in its anticancer mechanism (8, 9). The results in the present study were mixed, because not enough patients receiving ADR could be recruited. However, the enhanced excretion of K, P, and S after CP administration has not been reported before. The increased excretion of phosphorus indicates CP or phosphorus-containing metabolites of CP are present in the urine. Since personal baseline data were not available, the exact amount of phosphorus derived from CP could not be calculated.

Mutagenicity. The discussion of ADR and CP mutagenesis data for the pure compounds can be found elsewhere (34). The ADR and CP data for the reverse assay agreed generally with the results of Matney *et al.* (42). There appeared to be no synergism between CP and/or ADR and urine concentrate, contrary to the results of Gibson *et al.* (43).

The fact that TA1535-S9 shows a mutagenic response to the urine of CP-administered patients (S9 must be added for CP itself to show mutagenicity in this strain) implies that some CP metabolites are mutagenic without activation. Of the four urinary metabolites of CP [nornitrogen mustard, 4-keto-cyclophosphamide, 3-(2-chloroethyl)oxazolidone, and *N,N'*-bis (2-chloroethyl)-piperazine] tested by Balbinder *et al.* (44), nornitrogen mustard was the strongest mutagen, while 3-(2-chloroethyl)oxazolidone was the weakest. It was also possible that the presence of urine concentrate would enhance CP mutagenicity (43) but our experiments showed no synergisms between CP or ADR and urine concentrate or between spiked CP and ADR (34).

Two of the 11 cancer patients' urine samples tested in the reverse assay using strain TA1535 with S9 exhibited an increase in mutagenic activity over assays using the same strain without S9. Unmetabolized CP is known to be excreted in urine (12). Therefore, this increased mutagenic activity could be due to conversion of unchanged CP by the S9 to more mutagenic compounds. Further conversion of CP metabolites to more highly mutagenic metabolites may also occur. Balbinder *et al.* (44) observed that 4-keto-cyclophosphamide, which can act as a direct mutagen, showed an increase in mutagenic activity in the presence of S9. Individuals may vary in the proportions of the various metabolites formed. Relating the mutagenic activity in the urine concentrates from individuals to the dose of CP administered might then be confounded; correlational analyses involving explicit CP doses are also indicated (see below). CP is also not mutagenic to TM677-S9 but is with S9. ADR is mutagenic without S9. Again, some patients receiving CP without ADR showed mutagenesis for TM677-S9.

Urine samples collected from patients receiving cytostatis therapy have been shown to be mutagenic in other investigations (45–48). Nurses and pharmacy technicians handling cytostatic drugs also show increased urine mutagenicity (45, 49–52). Other studies have not confirmed these results (18, 46, 50, 53–55). In one study (53) only 2 days had elapsed from exposure to sampling time, and studies by Nguyen *et al.* (49) showed no significant increase in mutagenicity until the third day after exposure. In another study (46), samples were taken on Monday and Friday morning, rather than in the afternoon or as 24-h samples as for the studies showing positive results. Therefore, sampling strategies and the drug types administered, as well as different working conditions, may influence results.

Patient and Control Subject Information. In comparing the control subjects' and cancer patients' urinary GA, THIO, and mutagenicity results, age, sex, and medication differences between the groups must be considered. All but one of the cancer patients were female; 11 of the 15 control subjects were female. With drugs such as barbiturates, there are striking male-female differences in toxicity, due to levels of sex hormones (56) or increased fat sequestration. Since several of the cancer patients were also receiving steroids, such factors may be important.

Only MTX and 5-FU were given at concentrations high enough to be likely to perturb CP or ADR correlations. MTX and 5-FU are known to depress mixed function oxidase activities (57). VIN significantly increases the level of hepatic sulfatase at low doses given to rats and somewhat decreases it at higher doses (57). Lysosomal glucuronidase is substantially increased by VIN at high doses. Quinine (taken by one cancer patient, subject 2, for leg cramps) and quinidine (a cardiac antiarrhythmic taken by a control subject) have been shown by the antipyrine test to be microsomal enzyme inducers in humans (58). A dose-dependent reversible inhibition of the microsomal enzyme system in the liver of young irradiated animals has been shown (56). Several of the cancer patients reported receiving radiation treatment.

A low dose of prednisone (a steroid taken by one of the cancer patients) has been shown to cause a slight decrease in ethylmorphine demethylase in rats, with higher doses producing a marked decrease in specific activity of microsomal glucuronidase (57). Prednisone has been shown to be mutagenic (59). Procarbazine (an antineoplastic taken by one of the cancer patients) has been shown to be mutagenic, but only in bacterial strains not having the *rfa* mutation (60).

Several of the cancer patients and control subjects were taking diuretics. This would be expected to affect the concentration of drugs and CR excreted in the urine. The vomiting and diarrhea side effects of many of the antineoplastic drugs may also affect the excretory pattern of the drugs.

Several dietary factors (*e.g.*, fried beef meals, red wine, and grape juice) can influence the urinary mutagenicity assay system (61, 62). However, the contribution to urinary mutagenicity derived from dietary sources should be negligible compared to the mutagenic activity of CP or ADR, as shown by the control results in Table 6.

The activity of hepatic enzyme systems can vary with the nutritional status (*e.g.*, the decreased availability of the microsomal enzyme system in the protein- or metal-deficient animal) and emotional status of an animal (56). The cancer patients were also probably more stressed than the control subjects. Several of the cancer patients and one of the control subjects also took sedatives.

Most studies on the differences in toxicity based on age deal with immature *versus* adult comparisons. Effects of old age on the metabolism of toxic compounds has not been well studied. The population of control subjects was younger than the cancer patients; 73% of the cancer patients were older than 60 years old, whereas only 20% of the control subjects were over 60 years old. Neither group included anyone under the age of 32.

Therefore, the cancer patients and control subjects were not completely age, sex, disease, diet, and medication matched.

Data Analyses. The data analyses were conducted in three tiers, to mimic conventional epidemiology studies.

The first type of data analysis (Tables 4–6) simulates a study where the type of drug administered but not the dose is known, a commonly found situation that includes work practice data in occupational or environmental epidemiology studies. Thus, the data were subdivided into patients treated with CP without ADR, CP plus ADR, and all provided CP. Very little of the data were normally distributed [the exceptions were THIO controls (CR normalized), THIO after CP/ADR (daily), CR controls and CR (daily), and C, Mg, Na, P, S, and Sr controls). This might be expected if different administered doses perturb the exposure data (Tables 4–6). The significant ($P \leq 0.05$) comparisons relative to controls involved increased urine volume for CP without ADR; decreased CR (millimolar basis) for CP without ADR and all CP; decreased CR (daily) for CP/ADR; increased GA and THIO (on all bases); and increased K, P, and S for CP, and CP without ADR. Strains TA1535 (with or without S9) and TM677 (with or without S9) detected mutagenic urine after CP administration; TA98 (without S9), a frameshift detector, showed mutagenic urine after ADR administration. Therefore, parameters which show significant differences relative to controls are potential screening markers of the drugs administered in the present study. The pharmacokinetics of the drugs administered may be modified by the simultaneous administration of other drugs or by effects of disease (particularly liver and kidney). The toxicity of at least 11 commonly used anticancer drugs (including ADR) has been shown in animal studies to depend on time of administration (63). Also, circadian rhythms can affect the pharmacokinetics of the drugs administered (63).

The second type of data analysis using the same types of categories involves correlational regression of dependent variables with drug doses, without taking personal baseline data into account (Table 7). This is the most commonly encountered type of data analysis in the biological monitoring literature, because spot urine samples are usually taken without a prior baseline sample. Thus, these results will be discussed in detail.

GA increased when there was exposure to CP or CP/ADR, as shown by a significant increase in the GA group means for the treated cancer patients *versus* the control subjects (expressed both as data adjusted to CR and GA excretion per 24 h). Fig. 3 shows the CR-adjusted GA posttreatment levels and the dose of CP administered. After excluding the value for subject A [rejected with 90% confidence by the Q test (30)], a statistically significant ($0.01 < P < 0.05$) Pearson correlation coefficient of -0.904 was observed among the remaining 5 subjects treated with CP without ADR (Fig. 6). Among the five CP/ADR subjects, there was a statistically insignificant correlation ($r = -0.692$, 81% significance), as also noted for all CP-treated subjects ($r = -0.581$, 92%), as well as with ADR dose ($r = 0.581$).

The relationship between the 24-h GA excretion and CP dose for cancer patients shows similar results. A statistical significance is seen ($P < 0.01$) in the Pearson correlation coefficient ($r = -0.988$) for CP without ADR-treated subjects (again excluding subject A with 90% confidence by the Q test) and CP/ADR-treated subjects, when each group is considered separately, with a strong negative correlation; when data from all subjects are combined there is no statistical significance. No statistical significance was found in the correlation of 24-h GA excretion and ADR dose for cancer patients for subjects receiving ADR in combination with CP.

There is a negative correlation between CR-adjusted posttreatment THIO levels and the dose of CP administered (Fig. 7). The Pearson correlation coefficients were statistically significant ($0.01 < P < 0.025$) for the CP without ADR treatment (excluding data from subject 2 with 90% confidence by the Q test). However, neither the CP/ADR treatment group nor both treatment groups combined were statistically significant ($P > 0.05$). The negative correlation might indicate depletion of glutathione at the higher dose levels. For posttreatment THIO *versus* dose of ADR, no statistical significance was seen.

THIO and the corresponding GA in the 15 control subjects showed no statistically significant correlation (Pearson correlation coefficient, $r = 0.132$, $P > 0.05$). Likewise, the correlation for the posttreatment cancer patients (excluding one data point with 90% confidence by Q test) was not statistically significant (Pearson correlation coefficient, $r = 0.546$, $P > 0.05$).

The results in Table 7 for the conventional regression analysis of the entire data set yielded the following correlations ($r > 0.8$) for CP with ADR as independent variable; THIO, TM677 with or without S9, 24-h S9, and P. Similarly, with CP plus ADR as the independent variable, dependent variables were: GA, TM677 without S9, 24-h S9, Mg, Na, Si, and C. For all CP data, the dependent variables were: TM677 with or without S9 and 24-h S9, Na, and P.

The third type of data analysis was performed to account for any personal baseline factor. This is similar to pairing data when heterogeneous controls are present in a population, as, for example, accounted for by a paired Student t test (36). Thus, results for the elements, GA, and THIO may be very different, compared with those based on baseline uncorrected data. The mutagenicity data are already "paired," in the sense that all controls and preexposure specimens showed no mutagenicity and so there were no large baseline fluctuations. Unfortunately, establishing the latter fact about mutagenicity resulted in no personal baseline data for elements. This type of data analysis will now be considered in detail.

Firstly, it was important to establish that control 24-h and spot preexposure samples for patients were equivalent with respect to CR (millimolar basis). Since CR is used for normalization, any effects must be caused by the parameter of interest and not the normalizing variable. There were no significant differences between controls and preexposure CR. Therefore, comparisons between CR-normalized paired data are related to

Table 7 *Correlations ($r > 0.8$) and regression equations for all comparisons using non-baseline-corrected data*

CP, ADR, VIN, MTX, 5-FU, are in g. All other metals/independent variable comparisons are with the parameters normalized to creatinine (mg/mol creatinine for metals; μmol/g creatinine for GA and THIO. *S*, slope in appropriate units; *I*, intercept; *r*, correlation coefficient; RS, correlation coefficient significance. Subscript 24 signifies a 24-h parameter. TA1535 and TA98 results are in revertants/mmol creatinine; TM677 data are in revertants in 10^5 survivors/mmol creatinine.

		Without ADR				All				With ADR			
Dependent variable	Independent variable	*S*	*I*	*r*	RS (%)	*S*	*I*	*r*	RS (%)	*S*	*I*	*r*	RS (%)
GA	CP									−0.354	342	−0.688	80
GA	MTX	−0.0335	87.0	−0.965	95	−0.0335	87	−0.965	95				
GA_{24}	VIN	−46.7	142	−0.973	87								
	MTX	−0.253	80.5	−0.999	98								
THIO	CP	−0.0190	28.5	−0.625	81								
TM677	CP												
−S9						−0.767	960	−0.769	98	−0.244	557	−0.994	99
$-S9_{24}$						−4.64	5,862	−0.636	92	−14.7	2,971	−0.968	85
+S9		−1.73	2,057	−0.897	96	−1.24	1,574	−0.742	92				
$+S9_{24}$		−10.3	12,519	−0.880	95	−6.74	8,784	−0.826	99				
+S9	THIO	38.3	26.1	−0.686	80	47.3	17.1	0.829	97				
+S9	VIN					61.2	−170	0.999	97				
$+S9_{24}$	VIN					68.7	−1,537	0.989	90				
TA1535	ADR												
+S9										4.88	10,342	0.684	80
$+S9_{24}$										4.28	9,086	0.902	97
−S9	VIN												
+S9						−59.9	18,041	−0.878	88				
Metals						−65.5	19,434	−0.798	80				
Ca	5-FU					0.0144	−4.13	0.794	80				
	ADR	−0.249	25.8	−0.936	93					−0.249	25.8	0.936	93
	GA									3.70	0.0678	0.900	90
Fe										0.000121	0.00417	0.985	90
K	TM677 +S9	0.473	142	0.806	90	0.425	136	0.736	92				
Mg	CP									−0.232	23.4	−0.940	95
	ADR									−0.232	23.4	−0.940	95
	5-FU					−0.00695	10.8	−0.858	86				
	THIO					0.376	2.81	0.451	81				
	TM677 +S9					0.00916	3.23	0.631	82				
Na	CP					−0.499	978	−0.261	85	−2.72	246.3	−0.841	84
	ADR									−26.2	229.5	−0.966	97
	VIN					210	209	0.993	95				
	5-FU					0.468	−108	0.829	83				
	GA									7.26	−40.8	0.945	95
P	CP	−906	9,023.2	−0.940	99.5	−830	744,974	−0.846	99.5				
	MTX					−0.112	125	−0.975	85				
	THIO					4.87	61.9	0.463	82				
	TM677 +S9	0.103	73.8	0.719	83	0.0923	72.4	0.652	85				
	TA1535 +S9					0.00568	51.0	0.512	87				
S	MTX					−0.0284	126	−0.998	95				
	GA	−0.181	148	−0.753	91	−0.161	142	−0.527	87				
	TM677 +S9					0.114	83.4	0.602	80				
	TA1535 +S9					0.00610	62.4	0.472	83				
Si	CP									−0.00467	3.97	−0.950	95
	VIN					−0.223	0.808	−0.995	96				
	GA									0.0100	−0.149	0.859	85
	TA1535 −S9	38.2×10^{-6}	0.155	0.911	91								
	TA1535 +S9	30.9×10^{-6}	0.212	0.920	92					-189×10^{-6}	2.60	−0.810	81
Sr	THIO	−0.000308	0.0258	−0.494	85								
	TA1535 +S9	13×10^{-6}	0.00512	0.844	93	8×10^{-7}	0.0100	0.604	93				
	TA98 −S9									6.4×10^{-6}	0.0188	0.843	84
Zn	TA98 −S9									-25.3×10^{-5}	0.372	−0.879	88
C	CP									−28.9	23,771	−0.971	97
	5-FU					−1.51	2,400	−0.959	81				
	GA									68.4	−2,242	0.965	96
	TM677 −S9	33.7	−2,292	0.972	96								

the variable of interest. These results are provided in Table 8. This time, there is no GA *versus* CP correlation and GA is negatively correlated to MTX, with THIO also positively related to MTX and 5-FU. GA is positively correlated also with TM677 (with S9) data, as is GA for CP/VIN patients to TA1535 (with or without S9). THIO is related to TA1535 (with S9) response; CP/ADR causes a positive correlation but CP without ADR is negatively related.

For GA data (Fig. 3), there appears to be a post-/pretreatment GA ratio of 2 for each person. This explains the negative correlation found for the conventional analysis in Table 7 and implies that the system is saturated beyond a CP dose of approximately 3 mmol. For personal baseline-corrected data for CP with ADR, there was a negative Pearson correlation ($r = -0.930$) with increasing dose. There was no correlation for CP/ADR-exposed patients. This suggests an interaction of ADR with CP excretion.

The administration of a mean of 3.34 mmol CP resulted in a mean increase of 36.1 ± 18.4 μmol GA/g CR (posttreatment minus pretreatment GA excretion). The mean GA excretion for 15 control subjects was 31.7 ± 7.76 μmol GA/g CR. Clearly, paired data will lead to much greater sensitivity than uncorrected data, relative to individual signal/noise ratios. In that cancer patients before exposure to CP have individual GA values less than when they are exposed to CP, there may be a critical CP dose below which a dose response may be observed. Doses covered in this investigation ranged from 2.9 to 4.6 mmol CP; clearly, doses lower than 2.9 mmol should be used to define the critical saturating dose. For the treatment of cancer patients, exposures to high doses to CP are relevant, since

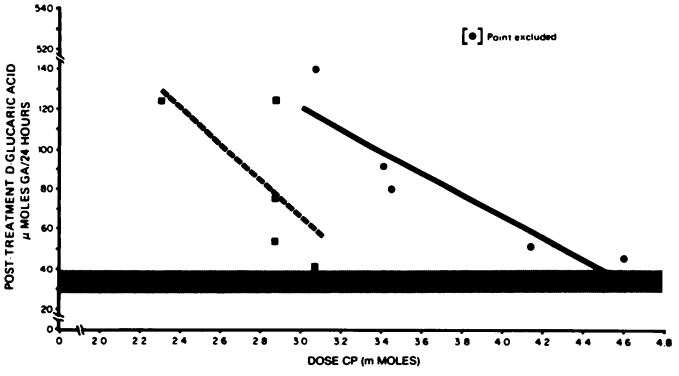

Fig. 6. Correlations between creatinine-adjusted posttreatment D-glucaric acid concentrations and cyclophosphamide dose for cancer patients. ●, cyclophosphamide treatment; ■, cyclophosphamide/adriamycin treatment. Shaded area indicates ± 1 SD from mean of control subjects.

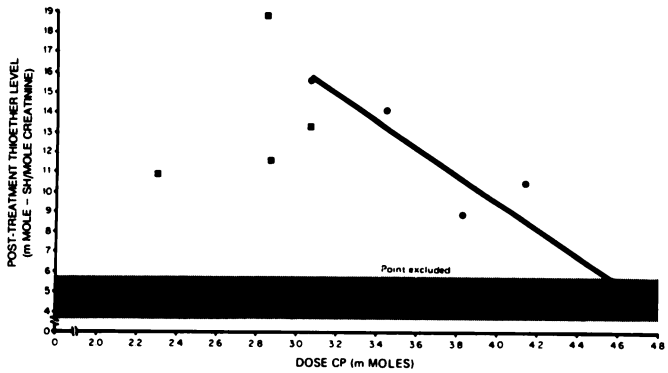

Fig. 7. Correlation between creatinine-adjusted posttreatment thioether levels and cyclophosphamide dose for cancer patients. ●, cyclophosphamide treatment; ■, cyclohosphamide/adriamycin treatment. Shaded area indicates ± 1 SD from mean of control subjects.

enzyme denaturation, saturation of various pathways, and liver or kidney toxicity may be related to drug side effects, and should be useful in the design of CP therapy and minimization of side effects.

Increases in GA excretion can be due both to increased microsomal enzyme induction and to conjugation with glucuronic acid. CP is metabolized by the mixed function oxidase system and is thus likely to induce these enzymes. However, acrolein, one of the metabolites of CP, denatures the mixed function oxidase enzymes (64) but is eventually excreted as a thioether (41). The dose and duration (acute or chronic) of CP exposure may then determine whether the net effect on the mixed function oxidase enzymes will be induction or denaturation. The cytochrome P-450-dependent mixed function oxidase system has been shown to be depressed following administration of ADR (65, 66). ADR presence could, therefore, reduce GA excretion. Several of the metabolites of CP and ADR have an —OH functional group. The Phase II conjugation of such compounds (*i.e.*, compounds having a nucleophilic functional group) to glucuronic acid would result in increased urinary excretion of glucuronides and GA, probably with different time courses.

The pre-/posttreatment comparison showed a statistically significant difference in the post- *versus* pretreatment CR-adjusted THIO levels for five of the eight subjects (Fig. 5). CP without ADR, CP/ADR, CP, and ADR doses did not correlate significantly with personal baseline-corrected THIO levels, although MTX and 5-FU did. There is an indication that the maximum post-/pretreatment THIO ratio is no greater than 4 but is more variable than for GA. Although rats excrete a specific thioether in their urine (41), the variable results of this study agree with those also reported on the variable levels of 3-hydroxypropylmercapturic acid of five patients (41). Since the average 24-h THIO excretion was 73.2 ± 28.6 μmol SH for CP-treated patients in this study, relative to 37.9 ± 19.5 μmol SH for controls, then 35.3 μmol SH/h is the THIO level due to CP exposure at these doses of the present study. As for the unpaired data, the corresponding GA and THIO data corrected for baseline are not correlated. This is not surprising, because the two represent different metabolic endpoints. However, the ratio of post-/pretreatment GA to the corresponding post-/pretreatment THIO is correlated (Pearson $r = 0.757$; $0.01 < P < 0.05$).

Because many environmental mutagens are xenobiotic compounds that require oxidation for detoxification, a relationship between body fluid mutagen levels and mixed function oxidase activity (one pathway for enzyme-mediated inactivation) has been proposed (26). The relationship between TM677 plus S9 mutagenicity and baseline-corrected GA is significant in the absence of ADR and for all CP-administered patients. The mutagenicity for TA1535 with or without S9 was observed for GA excreted by CP/VIN patients, indicative of a CP-VIN interaction. If CP itself is the drug causing mutagenesis, then the addition of S9 is required for mutagenesis to be correlated.

The tripeptide glutathione is implicated in the metabolism of activated electrophilic metabolites, which are postulated to be responsible for DNA damage, mutations, and ultimately cancer. Since thioethers are the end products of glutathione metabolism, it has been suggested that there is a correlation between THIO and urinary mutagenicity, and this has been observed in cigarette smokers (67). The reaction of activated electrophilic intermediates or stable electrophilic metabolites with glutathione can prevent covalent binding of the electrophiles to cellular macromolecules. In this way, glutathione conjugation represents a protective mechanism. Although glutathione conjugation generally results in the formation of thioethers that are less toxic than their parent electrophiles, a few exceptions are known in which glutathione conjugates have mutagenic properties, *e.g.*, for 1,2-dichloroethane and vicinal dihalogen compounds (68, 69). Only weak correlations were observed for

Table 8 *Correlations ($r > 0.8$) and regression equations for all comparisons using baseline-corrected data*

		Without ADR				All				With ADR			
Dependent variable	Independent variable	S^a	I	r	RS (%)	S	I	r	RS (%)	S	I	r	RS (%)
GA	MTX	−0.0212	45.8	−0.965	97	−0.212	45.8	−0.969	97				
THIO	MTX	0.00335	2.33	−0.907	91	0.00335	2.33	0.907	91				
	5-FU					0.0186	−13.9	0.984	90				
TM677 +S9	GA	14.2	−198	0.993	99	11.3	−6.66	0.807	90				
TA1535 +S9	THIO	−856	14,523	−0.702	81					384	9,805	0.971	86
TA1535 −S9	GA (CP+VIN)					−143	18,762	−0.970	88				
TA1535 +S9						−175	20,860	−0.998	95				

[a] S, slope; I, intercept; RS, correlation coefficient significance; r, correlation coefficient.

TA1535 plus S9 mutagenesis with CP without ADR (81%) and with CP/ADR (86%) for baseline-corrected THIO. It should be noted that only MTX and 5-FU, of the antineoplastic drugs, were correlated with baseline-corrected THIO. MTX also was inversely correlated with baseline-corrected GA. Hence, the data cannot be interpreted on the basis of CP and ADR doses alone.

Thus, as expected, after administration of many antineoplastic drugs and medications, the interpretation of results is difficult and subject to many unknown variables.

This is the first study to report simultaneous THIO, GA, mutagenesis, and elemental data for urine of patients administered antineoplastic drugs with real-life medications. It appears that the screening urinary measures best suited to monitor for extent of exposure to the antineoplastic drugs used in this investigation were the forward mutagenesis assay and D-glucaric acid levels.

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