Journal of Chromatography, 186 (1979) 659–676

CHROM. 11,953

IDENTIFICATION AND QUANTITATION OF NUCLEOSIDES, BASES AND OTHER UV-ABSORBING COMPOUNDS IN SERUM, USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. EVALUATION OF HUMAN SERA

RICHARD A. HARTWICK, ANTÉ M. KRSTULOVIC and PHYLLIS R. BROWN
Department of Chemistry, University of Rhode Island, Kingston, R.I. 02881 (U.S.A.)

SUMMARY

The reversed-phase mode of high-performance liquid chromatography was used to investigate the profiles of low-molecular-weight, UV-absorbing compounds in human serum. Identification techniques are described which allow for the identification of picomole amounts of the nucleosides, bases and other compounds in several microliters of serum ultrafiltrate.

The sera from 31 normal subjects (17 males, 14 females) showed very consistent profiles. A total of 12 compounds were identified and quantified in normal serum. The analysis of sera from over 150 patients with various types of neoplasia and other diseases showed serum profiles significantly different from normal profile.

INTRODUCTION

In recent years, efforts have been made to find biochemical markers for various diseases, especially neoplastic diseases. Such markers could be useful for early detection of the disease state or for confirmation of a diagnosis. In addition, it may be possible to use the markers to monitor the progress of the disease and the patient's response to therapy. The methylated nucleosides and bases, which have been found at elevated concentrations in the urine of patients suffering from several types of cancer, have been suggested as possible markers for leukemia, breast cancer and other malignancies[1–18]. Increased amounts of the modified nucleosides and bases have also been found in the urine of laboratory animals subjected to radiation[19,20]. It has been postulated by Borek and others[21,22] that increased excretion of the modified nucleosides and bases may reflect the turn-over rate of the tRNA, since many of these compounds have been found in this nucleic acid[22].

Although the nucleosides and bases in urine have been extensively studied, the presence of these compounds in serum has not been systematically investigated. Studies involving these compounds in serum have been hampered by the lack of an adequately sensitive methodology. The recent development of microparticulate chemically bonded packing materials in high-performance liquid chromatography

(HPLC) has made possible the routine analysis of the nucleosides and bases in biological samples such as serum[23–35].

Recent studies by several researchers have shown that certain of the nucleosides are present in detectable amounts in serum. For example, Agostoni *et al.*[26] and Rubio *et al.*[27] have found that serum concentrations of the deoxyribonucleosides and adenosine increase with myocardial infarction and reactive hypermia. Senftler *et al.*[28] have recently employed HPLC to study the serum as well as the hemodialyzate of uremic patients.

As nucleosides and bases have been found in detectable concentrations in human serum, it was felt that serum might serve as a useful physiological fluid in which to examine the overall nucleoside and base profiles and to study alterations of these profiles during neoplastic and other disease states.

As a first step in this long-range goal of studying alterations in serum nucleoside and base profiles in patients with cancer, it was necessary to determine the compounds which are found in normal serum and variations in these profiles due to sex, age, diet, etc. In addition, it was necessary to study effects of sample preparation and handling on the stability of these compounds. Only after these initial studies were done could the sera of patients with various types of malignant and non-malignant diseases be studied and the sera from healthy subjects be compared with those with disease states.

The purpose of our research was to determine the compounds found in normal sera and to investigate the sample handling techniques and identification procedures developed. In addition, preliminary results of studies involving patients with several types of cancer are reported.

MATERIALS AND METHODS

Instrumentation

The high-pressure liquid chromatograph (Waters ALC 204, Waters Assoc., Milford, Mass., U.S.A.) and the ancilliary equipment have been described in detail in Part I[38]. Instrumentation not discussed previously includes an on-line Model SF 770 UV–visible scanning spectrophotometer, with a model MM 700 memory module (Kratos, Schoeffel Instrument Division, Westwood, N.J., U.S.A.). The UV spectra were obtained using the stop-flow technique[29].

Columns

The reversed-phase (C_{18}) columns (30 cm × 4.6 mm I.D.) were obtained from Waters Assoc. Pre-columns (2.5 cm × 4.6 mm I.D.) used throughout the study were packed with pellicular C_{18} packing material (Whatman, Clifton, N.J., U.S.A.).

Reagents

Enzymes used in the enzymatic peak-shift identifications were of the highest purity available and were obtained from Sigma (St. Louis, Mo., U.S.A.). The chromatographic standards were also purchased either from Sigma or from Vega-Fox Biochemical Co. (Tuscon, Ariz., U.S.A.).

The $NaIO_4$ used for the determination of *cis*-diol group was purchased from Amend Drug and Chemical Co. (New York, N.Y., U.S.A.). KH_2PO_4 was of reagent

grade, obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). The spectral-grade methanol was from Burdick and Jackson (Muskegon, Mich., U.S.A.).

Normal reference sera (Seronorm) were obtained from Nyegaard (Oslo, Norway). Filter cones used for the ultrafiltration procedures were obtained from Amicon (Lexington, Va., U.S.A.). The nominal molecular weight limit of these filters was 25,000. Vacutainer serum tubes (no anticoagulant) were obtained from Beckton & Dickenson (Rutherford, N.J., U.S.A.).

Selection of normal subjects

Donors who had no apparent manifestations of disease were selected. Subjects were not maintained on a purine-free diet; however, histories were obtained concerning their dietary intake for at least 12 h prior to blood collection. Histories concerning vitamin intake, drug regimens, etc., were also noted.

Samples of patients with various diseases

Sera from cancer patients were obtained from the serum bank of the National Cancer Institute, at the Mayo Clinic, Rochester, Minn., through the courtesy of Dr. Ronald Herberman, Chief of NIH. Cancer samples were also obtained from Dr. James Crowley and Dr. Patricia Farnes at the Rhode Island Hospital.

Blood collection and handling

Sera were collected by subcubital veinipuncture into 10-ml (red top) Vacutainer tubes. Clot formation was allowed to proceed for 15–20 min at room temperature, after which the tubes were centrifuged at 1145 relative centrifugal force (RCF) for 5–10 min. Sera were then transferred to disposable plastic tubes and allowed to remain at room temperature (24–26°) for 3.5–4.0 h. They were then filtered through Amicon cone filters for about 20 min at 500 RCF. Sera filtrates were placed in polyethylene sample vials and stored at —20°.

Development of sample protocol

Effect of clot removal. The effects of variations in the handling procedures of serum samples were investigated. Two groups of samples were monitored at room temperature (25°) for periods of up to 10 h. One group was allowed to remain in contact with the clot while in the other group the sera were removed from the clots.

Effects of storage. The effects of storage conditions were examined by splitting a total of six sera samples into two groups, one control and one experimental. These sample pairs were further divided into two groups; one group of samples was frozen immediately after the blood clotted and the serum removed, and the other was incubated at 25° for 3.5–4 h (see Table III) before being frozen. The frozen samples were stored at —20° for periods up to 17 days. After thawing, the first set of samples was allowed to remain at 25° for 3.5–4 h, then processed. The second set was processed immediately upon thawing. All samples were compared with their controls and processed according to Table III. Thus, the experiment was designed to simulate two different handling procedures; one in which sera were stored frozen before the 4-h incubation time, and the other in which sera were incubated prior to freezing.

Chromatographic conditions

The optimization of the chromatographic conditions has been discussed in detail in Part I[38]. To summarize, the conditions selected were as follows: low strength eluent, 0.02 *M* KH_2PO_4, pH 5.6; high strength eluent, 60% methanol–water solution, degassed using a stream of helium gas; gradient, 0–100% of the high strength eluent in 87 min (linear gradient); flow-rate, 1.5 ml/min; temperature, ambient. Any combination of gradient times and methanol concentrations may be used to produce a gradient slope of 0.69%/min methanol increase.

Peak identification procedures

Spectral data. Absorbance ratios were obtained by measuring the peak height of a solute passing through two detectors in series using a dual-pen recorder. UV spectra were made using the stop-flow technique, over the range 220–320 nm. Fluorescence data were obtained at an excitation wavelength of 285 nm, with a cut-off filter of 320 nm. With the exception of the UV spectra, which required a separate injection, the detectors were in series so that pertinent spectral information could be obtained simultaneously in a single analysis using this bank of detectors.

Enzymatic peak shift. An aliquot of a sample (40–80 μl) was incubated at 25° with an equal volume of enzyme solution. The incubation time necessary is dependent upon the concentration of the solute in the sample and the enzyme activity of the solution. The enzymes commonly used in our laboratory for identification are listed in Table I together with the reaction conditions and necessary cofactors.

TABLE I

ENZYMES USEFUL FOR THE IDENTIFICATION OF SOME UV-ABSORBING COMPOUNDS FOUND IN HUMAN SERUM

Substrate(s)	*Reagent or cofactor*	*Enzyme*	*Reaction pH*	*Product(s)*
Hypoxanthine	H_2O, O_2	Xanthine oxidase (E.C. 1.2.3.2)	7.8	Xanthine
Xanthine	H_2O, O_2		7.8	Uric acid
Inosine	Phosphate	Purine nucleoside Phosphorylase (E.C. 2.4.2.1)	7.4	Hypoxanthine
Guanosine	Phosphate		7.4	Guanine
Guanine	H_2O	Guanase (E.C. 3.5.4.3)	8.0	Xanthine
L-Tryptophan	Pyridoxal-5-phosphate	Tryptophanase (E.C. 4.2.1.E)	8.3	Indole
Uric acid	H_2O, O_2	Uricase (E.C. 1.7.3.3)	8.5	Allantoin
Adenosine	H_2O	Adenosine deaminase (E.C. 3.5.4.4)	7.5	Inosine

After incubation, the enzyme was deactivated prior to re-chromatography of the incubation mixture by a brief exposure to a boiling water-bath. The minute amount of enzymic protein remaining in the sample was not removed.

Qualitative chemical tests. For the periodate reactions, 80-μl aliquots of the samples were reacted with equal volumes of 0.01 mole/l $NaIO_4$ solution. Reactions were complete within 15 min. If desired, the excess of periodate can be removed by the addition of ethylene glycol. Aliquots of the reaction mixture were injected directly on to the column after reaction with no apparent effects on column performance.

RESULTS

Chromatography and peak identification

Fig. 1 shows a chromatogram of a serum filtrate from a normal donor. The peaks which were present in the majority of the sera are lettered A–O. Fig. 1 is typical of the profiles which were observed for most of the normal sera. The peaks eluted prior to peak A are subject to interferences from a large number of com-

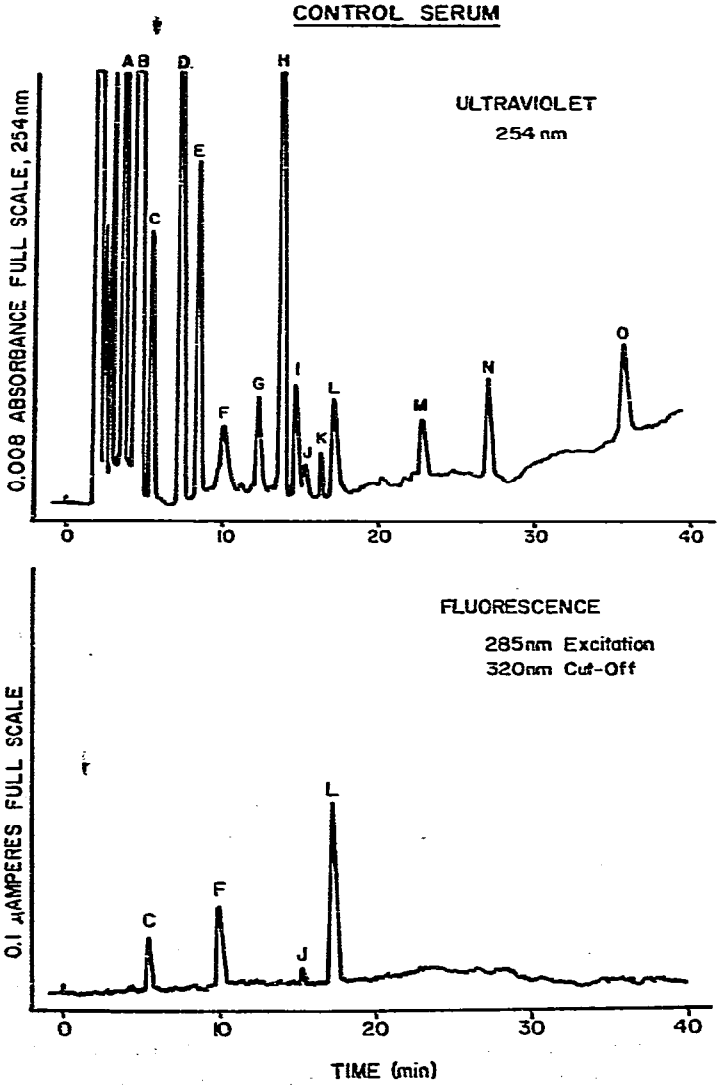

Fig. 1. Chromatogram of the serum filtrate from a normal donor using both UV (254 nm) and fluorescence (285 nm excitation, 320 nm cutoff) detection. Injection volume: 80 μl. Column: reversed-phase, 10-μm particle diameter. Eluents: low strength, 0.02 mole/l KH_2PO_4, pH 5.6; high strength, 60% methanol–water. Gradient: linear, 0–100% of high strength eluent in 87 min, slope 0.69% methanol/min. Flow-rate: 1.5 ml/min.

pounds not retained on a reversed-phase column. Therefore, no attempts were made to identify or quantify these peaks.

For preliminary identification, the retention times of the peaks A–O were compared to over 100 reference standards. For further identification, standards having retention times within 10% of the retention times of the serum peaks were co-injected with the serum (Fig. 2). The absence of any shoulders or unusual peak broadening indicates that the standards had the same retention times as the serum compounds.

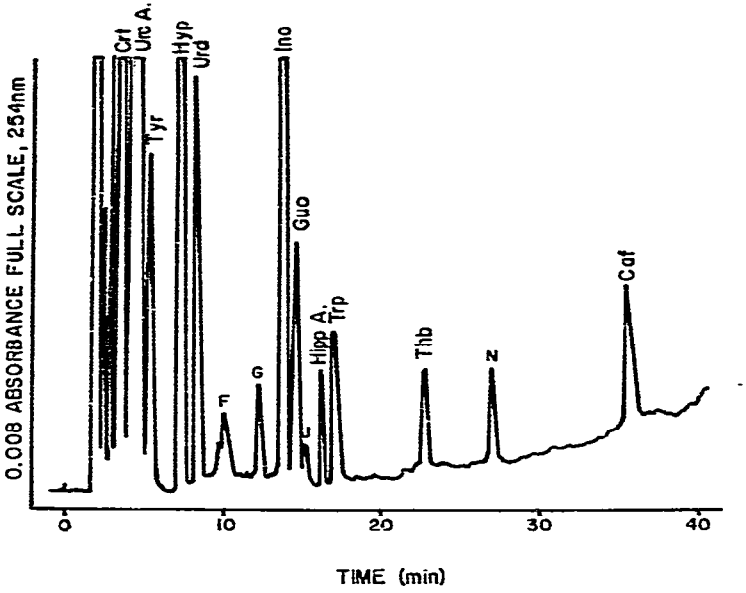

Fig. 2. Serum sample of co-injected with a solution containing creatinine, uric acid, tyrosine, hypoxanthine, uridine, inosine, guanosine, tryptophan, theobromine and caffeine.

The retention times of peaks labeled as F, G and J in Fig. 1 did not correspond to any of the reference standards. Peak F is composed of at least two co-eluting compounds. Peak J, which is present only occasionally, occurs in insignificant amounts. Peak G is consistently present in every normal serum sample. Its identity is still under investigation.

Peak height ratios. Peak height ratios were obtained on-line for every sample. These ratios can be useful for the identification of peaks and for the routine confirmation of peak purity[29]. Table II lists the peak height ratios of the serum peaks labeled in Fig. 1 as well as the ratios of the standards. Excellent agreement was found between both groups, with no significant differences between the two groups at the 95% significance level. The one exception is peak E, which showed a ratio intermediate between uridine and xanthine.

Fluorescence. The highly selective response of a fluorescence detector to the serum components is illustrated in the lower chromatogram of Fig. 1. Only four

TABLE II

COMPARISON OF AVERAGE PEAK HEIGHT RATIOS FOR SERUM PEAKS AND STANDARD COMPOUNDS

The lettered peaks correspond to those in Fig. 1. Numbers in parentheses are the standard deviations from five randomly selected samples. Calculated t values using the two-group comparison test are reported at the 95% confidence level.

Serum peak	*Peak height ratio (1 S.D.)*	*RSD (%)*	*Standard*	*Peak height ratio (1 S.D.)*
A	V.S.*	—	Creatinine	0.001*
B	2.21 (0.184)	8.33	Uric acid	2.16 (0.104)
C**	2.42 (0.0592)	2.44	Tyrosine**	2.47 (0.0800)
D	0.0425 (0.00191)	4.49	Hypoxanthine	0
E	0.278 (0.0254)	9.14	Uridine	0.215 (0.0120)
			Xanthine	0.459 (0.0100)
G	0.375 (0.0286)	7.63	—	—
H	0.0955 (0.0102)	10.7	Inosine	0.0920 (0.0067)
I	0.386 (0.0406)	10.5	Guanosine	0.373 (0.0200)
K	V.S.*	—	Hippuric acid	0.001*
L**	1.48 (0.0859)	5.80	Tryptophan**	1.43 (0.0480)
M	1.26 (0.0239)	1.90	Theobromine	1.27 (0.0290)
O	1.28 (0.0801)	6.23	Caffeine	1.26 (0.0561)

* V.S.: peak height ratio is too small to measure accurately.
** Indicates that the peak was fluorescent at 285 nm excitation, 320 nm cutoff filter.

major fluorescent peaks, C, F, J and L, were found in the serum of subjects with no known disease. Of these, only peaks C and L have been unambiguously identified as being L-tyrosine and L-tryptophan, respectively.

UV spectra. The UV absorption curves for several of the serum peaks and reference standards are shown in Fig. 3. These spectra were obtained using the stop-flow techniques described earlier[30]. The agreement of spectral details between the serum peaks and the standards is evident from Fig. 3.

The UV spectra of peak E in Fig. 1 is compared with both uridine and xanthine in Fig. 3 since both of these standards had retention times similar to that of peak E. Close examination of the spectra shows that the λ_{max} of peak E is similar to that of uridine, but shifted bathochromically towards that of xanthine. From the peak height ratios and from the complete UV curves, it is evident that peak E is composed of a mixture of both xanthine and uridine.

The UV spectrum of peak G is presented in Fig. 4. This peak, which appears normally in every normal serum sample and is elevated occasionally in serum of patients with chronic lymphocytic leukemia, has not yet been identified. Initial studies using both chemical ionization and electron-impact ionization mass spectrometry have been inconclusive. However, further mass spectral studies are currently underway in an attempt to identify this compound.

Enzymatic peak shifts. The identification of compounds in biological samples can be aided by the use of the enzymatic peak shift[29]. In this technique, the disappearance of a peak and/or the appearance of new peaks can be used to indicate that an enzyme-catalyzed reaction has occurred.

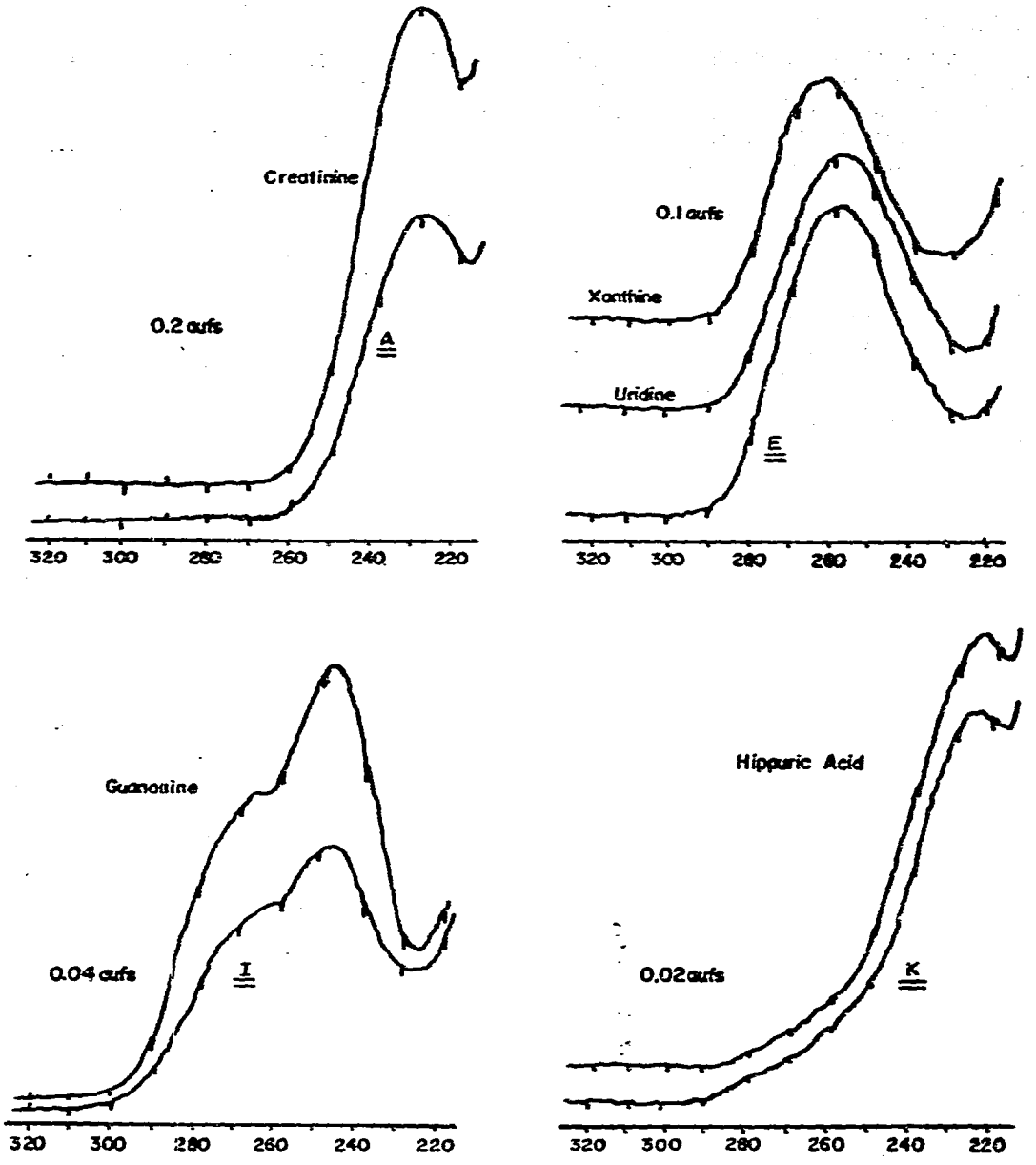

Fig. 3. UV spectra of several of the serum peaks identified in Fig. 1, obtained using the stopped-flow technique. Sensitivities are indicated in the individual spectra.

The following enzymes were used to confirm the peaks identified in Figs. 1 and 2; uricase for uric acid; tyrosinase for tyrosine; xanthine oxidase for hypoxanthine and xanthine; purine nucleoside phosphorylase for inosine and guanosine; tryptophanase for tryptophan; and adenosine deaminase for adenosine. Although

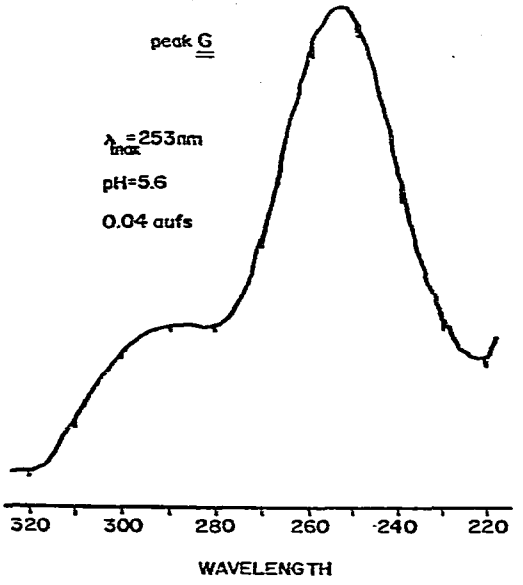

Fig. 4. UV scan of peak G in Fig. 1.

the technique is usually used qualitatively, enzyme-catalyzed reactions can be used for quantitation if proper care is taken.

Chemical tests. Many classical qualitative organic tests are compatible with HPLC and can be used to determine the presence of functional groups in compounds at the picomole level. Aliquots of the samples are reacted in a micro-vessel. A change in retention times or the disappearance of a peak is taken as an indication of a reaction. The periodate reaction, which is highly specific for any *cis*-diol groups, was found to be useful for the identification of compounds containing the ribosyl moiety. Fig. 5 shows the serum sample presented in Fig. 1 after reaction with periodate. The peaks labeled as H and I (Ino and Guo) are completely removed, indicating the absence of any co-eluting compounds not containing the diol group. The reaction products are eluted in the first part of the chromatogram.

Peak E in Fig. 1 is seen to decrease in Fig. 5 by 50–70% after reaction with periodate. From the UV spectra and retention data, it has been found that peak E consists of both uridine and xanthine. The periodate reaction was used to determine quantitatively the amounts of uridine by subtraction of peak areas after reaction.

Use of combined data for peak identification. The peak identification procedures outlined on previous pages, while not conclusive in themselves, can be very powerful when used in combination. Thus a sequence of tests can be used in place of a single more definitive method (such as mass spectral data) when the sample amounts preclude the collection of sufficient amounts for such analyses.

Development of sample protocol

Fig. 6 shows the changes in areas of serum peaks over a 10-h period at 25°. Substantial changes in several of the peaks occur during the first several hours before sample processing. Most of these changes are probably enzymatically catalyzed. For

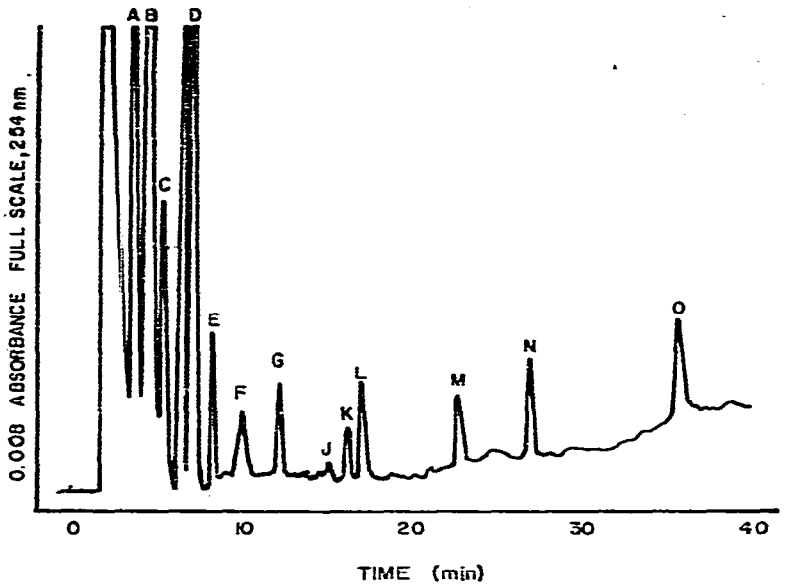

Fig. 5. Chromatogram of 80 μl of the same sample as in Fig. 1, after reaction with 80 μl of 0.01 mole/l $NaIO_4$ solution. Total injection volume: 160 μl.

example, the rapid decrease in adenosine is catalyzed by endogenous adenosine deaminase. Inosine (the product of adenosine) increases simultaneously, and then later decreases. Hypoxanthine, as the product of the purine nucleoside phosphorylase deribosylation of inosine, also increases after a short lag time.

Peak F (Fig. 1) decreases rapidly during the first 2–3 h, after which it remains constant at the concentration shown in Fig. 1. Although peak F is not positively identified as yet, from preliminary work the compound appears to contain adenine and is tentatively identified as NADH.

Creatinine and uric acid remain essentially constant throughout the 10-h period, along with peak G, theobromine and guanosine.

The peaks in the chromatograms of sera which was left over the clot showed similar changes to those presented in Fig. 6. The major difference was in the inosine concentration, which increased substantially after 4–6 h.

Although there were considerable interconversions in the peaks in the serum profiles, it is important to note that no new large peaks were found during the 10-h studies. Occasionally, two small peaks were observed when the sera had remained at room temperature for more than 5–6 h. These peaks eluted just after tyrosine and immediately before inosine. The absence of significant qualitative changes is important in the cancer studies, where the appearance of new compounds, rather than changes in the concentrations of existing compounds, might be used to indicate the presence of neoplastic changes. Following these studies, the protocol presented in Table III was adopted for all subsequent work.

Effect of storage conditions

Serum samples which were frozen immediately after collection for periods of

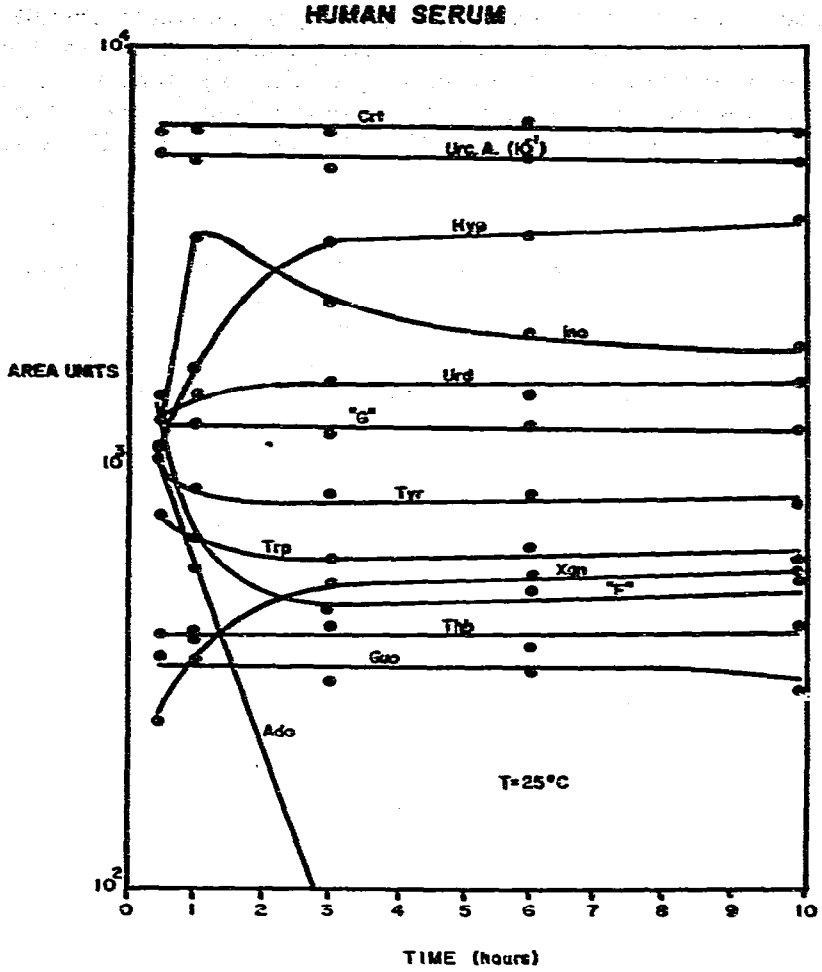

Fig. 6. Changes in peak areas of a serum sample incubated at 25° (removed from the clot) for periods of up to 10 h. Curves represent the serum peaks lettered in Fig. 1.

TABLE III

SUMMARY OF STANDARD PROTOCOL CONDITIONS ADOPTED FOR PROCESSING AND STORAGE OF SERUM SAMPLES PRIOR TO ANALYSES BY HPLC

Step	*Procedure*
1	Collect blood in serum (red top) tubes.
2	After clotting (15–20 min at 25°), centrifuge for 15–20 min at 1145 RCF.
3	Withdraw serum into a plastic vial. Incubate at 25° for 3.5–4.0 h.
4	Add internal standard if used.
5	Place sample in Centriflo membrane filter (previously soaked in distilled water for 1 h).
6	Centrifuge for 20 min at 500 RCF.
7	Remove filtrate.
	Freeze immediately at −20°.

up to 21 days and then processed showed no significant changes over control samples which were processed immediately according to Table III. Therefore, whole sera may be stored frozen without substantial changes, as long as the total time spent at 25° remains constant. However, it would appear to be more reliable to process the sera *in situ* without prior storage according to the protocol of Table III where possible.

Reproducibility

The reproducibility of peak areas was investigated by performing replicate analyses of individual samples of serum filtrate. The results of this study are shown in Table IV. For compounds found in the serum at relatively high concentrations, *e.g.*, uric acid, the average relative standard deviation averaged between 1 and 3%. For peaks of smaller areas, the precision was in the range of 3–6% RSD.

TABLE IV

PRECISION OF QUANTITATION FOR A SINGLE NORMAL SERUM FILTRATE

Five consecutive 80-μl injections.

Compound	$\bar{X}$ *(μmole/l)* *	*RSD (%)*
Creatinine	76.4 (2.38)	3.11
Uric acid	221 (2.92)	1.32
Tyrosine	70.4 (4.31)	6.13
Hypoxanthine	7.69 (0.0919)	1.20
Inosine	4.70 (0.164)	3.49
Guanosine	0.544 (0.0273)	5.01
Tryptophan	14.2 (0.653)	4.60

* Standard deviations for five replicate injections are given in parentheses.

UV-absorbing compounds in normal serum

A tabulation of the average concentrations of the compounds identified in the sera of 31 normal donors (14 females, 17 males) is presented in Table V.

Most of the nucleosides and bases found are present at concentrations between 0.001 and 0.1 times that of compounds such as uric acid and creatinine which are commonly analyzed in the clinical laboratory. Concentrations of dietary compounds such as theobromine and caffeine are also listed in Table V. Where possible, literature values for compounds of interest have been compared. The agreement between the literature values and those obtained here indicate the general reliability of the methodology used.

In order to confirm the accuracy of the sample preparation and HPLC procedures, commercially available standard reference sera were analyzed. Table VI shows the results of the average values obtained from three independent reference sera. The HPLC technique averaged 6.96% lower for the creatinine than the values obtained by the Jaffé method (reference sera) and 12.0% lower for the uric acid than the values obtained by the uricase method. The lower concentration for creatinine measured by HPLC is in agreement with that of other researchers[35], who have found that the widely used Jaffé alkaline picrate method is subject to interferences from other serum compounds.

TABLE V

COMPOUNDS IDENTIFIED IN NORMAL HUMAN SERUM

Sera were processed by membrane centrifuge cones (nominal molecular weight 25,000). Means represent 31 donors (17 males, 14 females). N.A. = Not available.

*Compound**	*Sex*	*$\bar{X}$ (μmole/l)***	*Range (2 S.D.)*	*Literature values (μmole/l)****	*Reference*
Creatinine	F	63.4 (8.7)	46.0–80.8	71–106	31
(A)	M	83.1 (11.8)	59.5–107	80–133	31
Uric A	F	171 (30)	110–232	155–357	31
(B)	M	295 (39)	217–373	208–429	31
Tyr	N.S.§	62.2 (16.3)	29.6–94.8	44–71	31
(C)				71–(4)	32
Hyp	N.S.	7.16 (2.81)	1.56–12.8	3.08–19.0 (male)	33§§
(D)				1.84–2.5 (female)	
Urd (E)	N.S.	3.17 (1.11)	0.951–5.39	N.A.	—
Xan (E)	N.S.	2.62 (1.04)	0.542–4.70	1.64	—
Ino (H)	N.S.	5.62 (2.87)	0.0–11.4	N.A.	—
Guo (I)	N.S.	0.881 (0.515)	0.0–1.98	N.A.	—
Hipp A. (K)	N.S.	0.613 (0.477)	0.0–1.57	N.A.	—
Trp (L)	N.S.	13.7 (3.57)	6.63–20.8	17–(2)	34
				9.16–(10.4)	35
Dietary compounds					
Thb (M)	—	—	0.0–6.35	N.A.	—
Caf (O)	—	—	0.0–12.2	N.A.	—

* Letters correspond to the identification of serum peaks given in Fig. 1.

** Standard deviations of triplicate analyses are given in parentheses.

*** All literature values have been converted to micromoles per liter. Standard deviations are given in parentheses. Ranges are indicated by dashes.

§ No significant sex-related differences.

§§ Other literature values are presented in this paper.

TABLE VI

COMPARISON OF CREATININE AND URIC ACID CONCENTRATIONS IN THREE REFERENCE SERA SAMPLES, ANALYZED BY HPLC AND BY THE JAFFE' METHOD (CREATININE) AND URICASE/PHOTOMETRY (293 nm) METHOD

Rereference sera were from Nyegaard (Oslo, Norway) (Seronorm, batch 136).

Compound	*$\bar{X}$ (μmole/l)**		*Difference (%)*
	HPLC method	*Seronorm*	
Creatinine	107 (3)**	115 (6)	6.96
Uric acid	428 (5)	440 (12)	12.0

* The means represent three independent samples.

** Standard deviations are given in parentheses.

The reproducibility of the profiles and the fact that the same peaks were found to occur in all of the 31 normal samples is important in our work. Normal dietary variations and vitamin intake did not alter the serum profiles to any large

extent, except for the compounds caffeine (peak O), theobromine (peak M) and an apparent caffeine metabolite (peak N). The range of variations that did occur are presented in the data in Table V. It should be noted that although the values for the concentrations of the UV-absorbing compounds in serum are highly reproducible, a normal range of values which is statistically valid can only be obtained with a much larger sampling (300–500 samples)[37]. Work is now in progress to establish these values.

Analysis of sera from patients with neoplasia and other diseases

In preliminary work, the sera from a total of approximately 150 patients with various types of cancer have been examined. Those types of cancer which produced the greatest changes in serum profiles were examined in more carefully controlled studies. The preliminary results of several of these studies are presented below.

Fig. 7 shows the chromatogram of the serum ultrafiltrate from a patient with chronic renal failure and chronic lymphocytic leukemia (CLL). The tremendous changes over the normal profile shown in Fig. 1 are evident, and are similar to those alterations observed by Senftler *et al.*[28]. Most of the compounds in Fig. 7 have not been identified, although many are highly fluorescent. The profiles of 21 patients with CLL above were examined in a seprate study. The CLL profiles were very similar to the normal profile shown in Fig. 1, with only one compound (peak G) being elevated. Thus, most of the changes observed in Fig. 7 appear to be due to a build-up of compounds from the renal failure, rather than from leukemia.

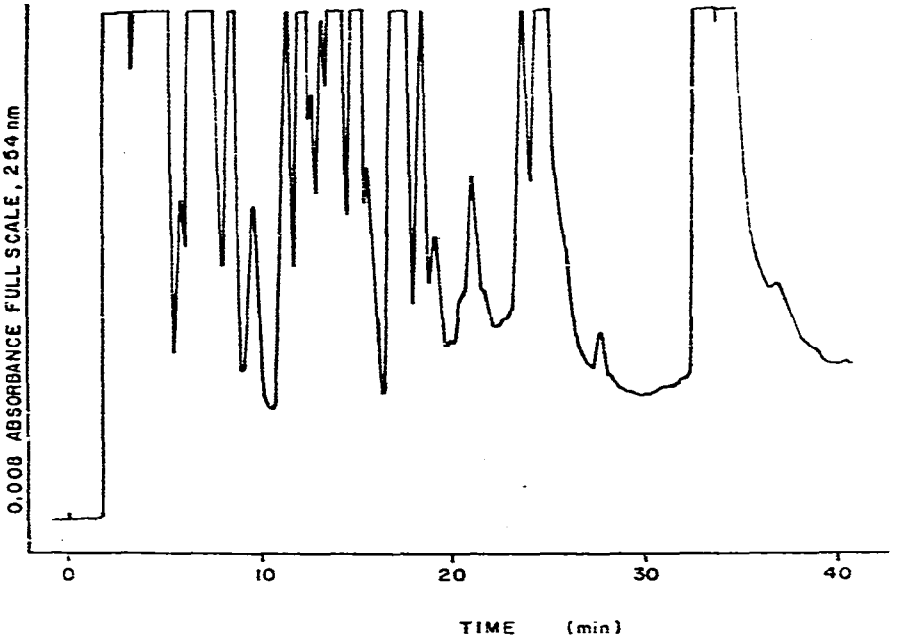

Fig. 7. Chromatogram of the serum ultrafiltrate from a patient with chronic renal failure and chronic lymphocytic leukemia (CLL). Injection volume: 80 μl. Chromatographic conditions as in Fig. 1.

Fig. 8 presents a serum profile of a 46-year-old male with malignant lung cancer. This profile was typical of the 16 lung cancer patients studied. The peaks labelled in parentheses (1-m-Ino and N_2-m-Guo) have been tentatively identified by retention times only, and are subject to further verification.

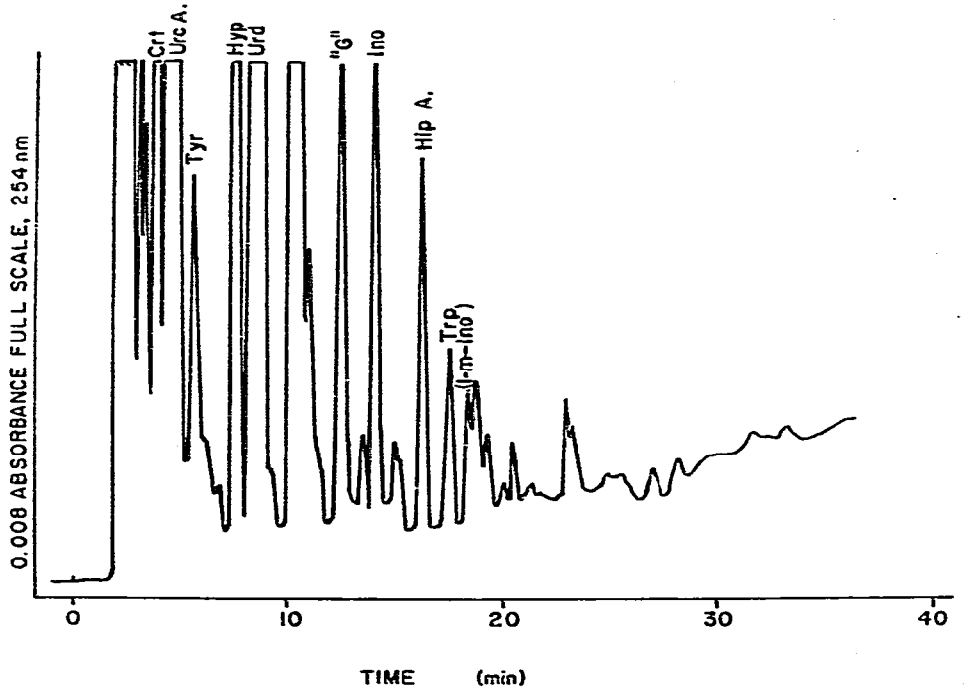

Fig. 8. Chromatogram of the serum ultrafiltrate of a 46-year-old male patient with malignant lung cancer. Injection volume: 80 μl. All other conditions as in Fig. 1. Peaks identified with parentheses represent tentative identifications by retention times and are subject to confirmation by other techniques.

A study of 13 patients with malignant breast cancer and 9 patients with benign fibrocystic changes was undertaken. Fig. 9 shows the chromatogram of the serum profile of a breast cancer patient with bone metastasis. Two new peaks not found in normal sera were observed in the serum shown in Fig. 9. These peaks have been tentatively identified as 1-m-Ino and N_2-m-Guo. In all, 45.5% of the malignant breast cancer patients exhibited significant concentrations of 1-m-Ino, while 22.7% had detectable concentrations of N_2-m-Guo[39]. Examination of 9 patients with benign fibrocystic changes showed essentially a normal serum profile, with no detectable amounts of 1-m-Ino or N_2-m-Guo[39].

From these and other studies, it is evident that alterations in the serum nucleoside and base profiles do occur during some types of cancer. However, these results are preliminary and it remains to be seen if specific changes can be consistently correlated directly to neoplastic diseases. As yet, it has not been proven that the

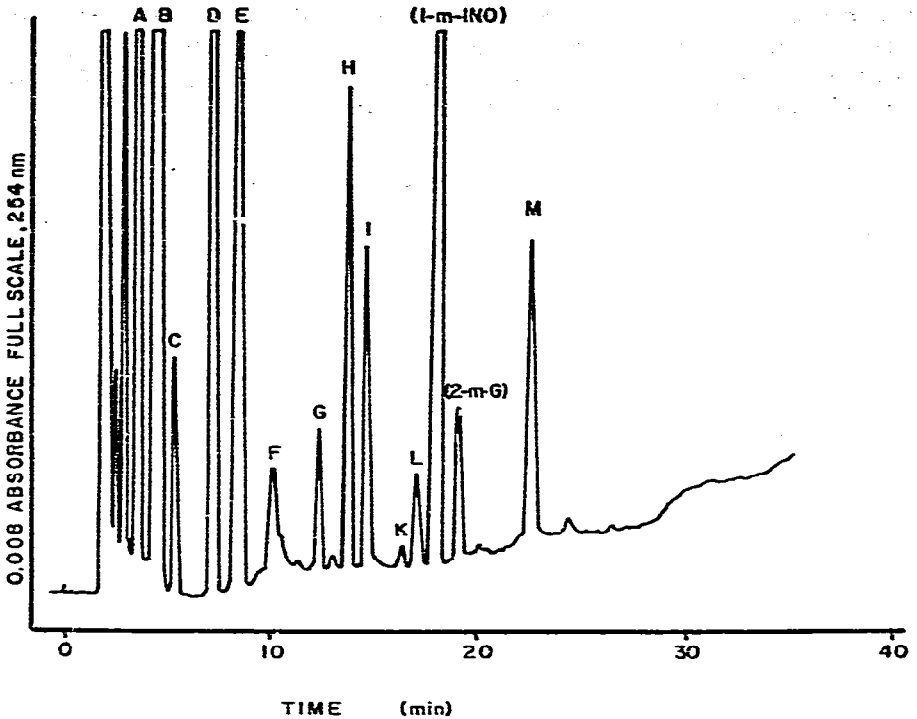

Fig. 9. Serum profiles of a post-operative breast cancer patient with metastasis to the bone. Injection volume: 80 μl. Peaks identified with parentheses are tentatively based upon retention times alone.

changes observed are disease-specific and if they are, at what stage of the disease such changes are first detectable. More comprehensive studies are currently underway in our laboratory concerning these changes.

DISCUSSION

The changes in the serum in profiles of the low-molecular-weight, UV-absorbing compounds caused by cancer or other diseases has received relatively little interest. However, our preliminary investigations indicate that not only are the normal profiles consistent but that definite changes occur in sera of patients with several types of cancer. It should be emphasized, however, that at the present time variations in HPLC profiles cannot be used to denote the presence of a neoplastic state. It remains to be seen how consistent these changes are for large populations, the sensitivity of the changes to the earliest stages of a disease, and whether the changes noted are disease-specific.

The HPLC methodology developed for this work is simple and precise with a minimum of sample preparation steps required. Studies of the various sample handling and storage techniques have indicated that the serum should be properly handled for accurate quantitation. However, small variatiosn in the handling procedures did not alter the qualitative profiles of the normal sera. In addition, the

relatively general freedom from interferences permits sensitive detection of new compounds as well as substantial changes in the concentrations of existing compounds in serum.

ACKNOWLEDGEMENTS

The authors thank Dr. James Crowley, Special Haematology, Rhode Island Hospital, and Dr. Ronald Herberman, Chief of Immunodiagnosis Laboratory at the National Cancer Institute, for the sera of the cancer patients. Thanks are given to Mr. John Rivers and the staff at the University of Rhode Island Health Services for their cooperation in the procurement of normal sera. Acknowledgement is extended to Mr. Malcolm McKeag and Mr. Sebastian Assenza for their general assistance with the laboratory work involved.

Appreciation is also extended to Paul Champlin (Waters Assoc.), Klaus Lohse (Schoeffel Instruments, Kratos) and Fred Rabel (Whatman) for their technical assistance.

Support for this work was provided in part by an American Chemical Society Summer Analytical Division Fellowship, and by Grant No. 17603 from the National Cancer Institute.

REFERENCES

1 G. E. Davis, R. D. Suits, K. C. Kuo, C. W. Gehrke, T. P. Waalkes and E. Borek, *Clin. Chem.*, 23 (1977) 1427.
2 M. Uziel, L. H. Smith and S. A. Taylor, *Clin. Chem.*, 22 (1976) 1451.
3 T. P. Waalkes, C. W. Gehrke, R. W. Zumwalt, S. Y. Chang, D. B. Lakings, D. C. Tormey, D. L. Ahmann and C. G. Moertell, *Cancer*, 36 (1975) 390.
4 T. P. Waalkes, C. W. Gehrke, W. W. Bleyer, R. W. Zumwalt, C. L. M. Olweny, K. C. Kuo, D. B. Lakings and S. A. Jacobs, *Canc. Chemother. Rep.*, 59 (1975) 721.
5 T. C. Tormey, T. P. Waalkes, D. Ahmann, C. W. Gehrke, R. W. Zumwalt, J. Snyder and H. Hansen, *Cancer*, 35 (1975) 1095.
6 J. Mrochek, S. R. Dinsmore and T. P. Waalkes, *J. Nat. Cancer Inst.*, 53 (1974) 1553.
7 G. B. Chheda, A. Mittelman and J. R. Grace, Jr., *J. Pharm. Sci.*, 58 (1969) 75.
8 K. P. Heirwegh, C. Ramboer and J. De Groote, *Amer. J. Med.*, 42 (1968) 913.
9 K. Fink and W. S. Adams, *Arch. Biochem. Biophys.*, 126 (1968) 27.
10 E. S. McFarlane and G. J. Shwa, *Can. J. Microbiol.*, 14 (1968) 185.
11 A. Dlugajczyk and J. J. Eiler, *Proc. Soc. Exp. Biol. Med.*, 123 (1966) 453.
12 L. R. Mandel, P. R. Srinivasan and E. Borek, *Nature (London)*, 209 (1966) 586.
13 S. M. Weissman, M. Lewis and M. Karon, *J. Hematol.*, 22 (1963) 657.
14 K. Fink, W. S. Adams, F. W. Davis and M. Nakatani, *Cancer Res.*, 23 (1963) 1824.
15 R. W. Park, J. F. Holland and A. Jenkins, *Cancer Res.*, 22 (1962) 59.
16 W. S. Adams, F. Davis and M. Nakatani, *Amer. J. Med.*, 28 (1960) 726.
17 M. Adler, *Science*, 130 (1959) 862.
18 B. Weissmann, P. A. Bromberg and A. B. Gutman, *J. Biol. Chem.*, 224 (1957) 407.
19 I. W. Chen, J. G. Kereiakes, B. I. Friedman and E. L. Saenger, *Radiology*, 91 (1968) 343.
20 C. D. Guri and L. J. Cole, *U.S. Navy Radiol. Defence Lab.*, 12 April (1968) 1.
21 E. Borek, *Cancer Res.*, 31 (1971) 596.
22 A. E. Pegg, *Advan. Cancer Res.*, 25 (1977) 195.
23 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
24 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 150 (1978) 455.
25 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
26 A. Agostoni, B. Lomanto and L. Natale, *Acta Cardiol.*, 22 (1967) 362.

27 R. Rubio, R. M. Berne and M. Katori, *Amer. J. Physiol.*, 216 (1969) 56.
28 F. C. Senftler, A. G. Halline, H. Veening and D. A. Dayton, *Clin. Chem.*, 22 (1976) 1522.
29 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
30 A. M. Krstulovic, R. A. Hartwick, P. R. Brown and K. Lohse, *J. Chromatogr.*, 158 (1978) 365.
31 N. W. Tietz (Editor), *The Fundamentals of Clinical Chemistry*, Saunders, Philadelphia, 2nd ed., 1976, pp. 1206–1226.
32 R. H. McMenamy, C. C. Lund and J. L. Oncley, *J. Clin. Invest.*, 36 (1957) 1672.
33 H. Pfadenhauer, *J. Chromatogr.*, 81 (1973) 85.
34 R. H. McMenamy, C. C. Lund, J. Van Marcke and J. Oncley, *Arch. Biochem. Biophys.*, 93 (1961) 135.
35 A. M. Krstulovic, P. R. Brown, D. M. Rosie and P. B. Champlin, *Clin. Chem.*, 23 (1977) 1984.
36 W. L. Chiou, G. W. Penz, M. A. F. Gadalla and S. T. Neremburg, *J. Pharm. Sci.*, 67 (1978) 292.
37 H. F. Martin, B. J. Gudzinowicz and H. F. Fanger, *Normal Values in Clinical Chemistry*, Marcel Dekker, New York, 1975.
38 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.
39 A. M. Krstulovic, R. A. Hartwick and P. R Brown, *Clin. Chim. Acta*, in press.