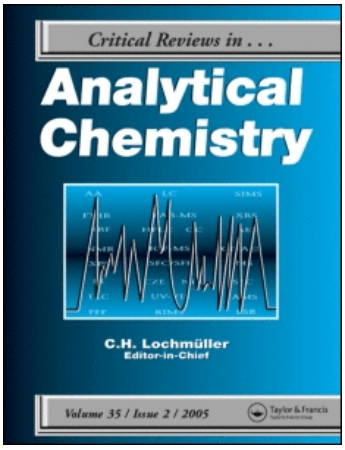

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

High Pressure Liquid-Chromatographic Analysis of the Nucleosides, Bases, and Other UV Absorbing Compounds in Biological Fluids

R. A. Hartwick; P. R. Brown; Charles W. Gehrke

To cite this Article Hartwick, R. A. , Brown, P. R. and Gehrke, Charles W.(1981) 'High Pressure Liquid-Chromatographic Analysis of the Nucleosides, Bases, and Other UV Absorbing Compounds in Biological Fluids', Critical Reviews in Analytical Chemistry, 10: 3, 279 — 337
To link to this Article: DOI: 10.1080/10408348108542728
URL: http://dx.doi.org/10.1080/10408348108542728

HIGH PRESSURE LIQUID-CHROMATOGRAPHIC ANALYSIS OF THE NUCLEOSIDES, BASES, AND OTHER UV ABSORBING COMPOUNDS IN BIOLOGICAL FLUIDS

Authors: R. A. Hartwick
Department of Chemistry
Rutgers University
New Brunswick, New Jersey

P. R. Brown
Department of Chemistry
University of Rhode Island
Kingston, Rhode Island

Referee: Charles W. Gehrke
Experiment Station Chemistry Laboratories
University of Missouri
Columbia, Missouri

TABLE OF CONTENTS

I. Introduction
- A. Scope of the Review
- B. Nomenclature of the Nucleic Acid Components
  - 1. Naming the Aglycones
  - 2. Naming the Sugars
  - 3. Tautomeric Forms
- C. Physical Properties
- D. Biochemical Significance of Nucleosides and Bases

II. Chromatography of the Nucleosides and Bases
- A. Ion Exchange
- B. Ion Exclusion
- C. Gel Packings and Miscellaneous Techniques
- D. Chemically Bonded Microparticulate Packings
  - 1. Ion Exchange
  - 2. Reversed Phase
    - a. Effect of Organic Modifier
    - b. Effect of pH
    - c. Gradient Elution
    - d. Gradient Optimization
      - i. Derivation of Retention Equations
      - ii. Application of Retention Equations
    - e. Reproducibility of Retention Times
    - f. Quantitation and Lower Detection Limits

III. Sample Preparation
- A. Review of the Methods
- B. Evaluation of Methods
  - 1. Borate Columns

2. Comparison of Techniques
a. TCA
b. Ammonium Sulfate
c. Ultrafiltration
d. Reversed-Phase Cartridges
3. Efficiency of Protein Removal
C. Conclusions

IV. Peak Identification
A. Retention Data
B. Spectral Data
1. Absorbance Ratios
2. UV Spectra
3. Fluorescence
C. Enzymatic Peak Shifts
D. Chemical Derivatization

V. Applications
A. Biochemical Markers
1. Urine
2. Serum
B. Dialysates
C. Metabolic Studies
D. Adenosine
E. Nucleoside Analogs

VI. Conclusions and Future Trends

References

I. INTRODUCTION

A. Scope of the Review

Nucleic acid components play central roles in biochemistry. The purine and pyrimidine base pairs are the chemical basis of the nucleic acids and their composition is of basic interest in genetic and other biomedical studies. In addition, nucleotides, nucleosides, or their bases are important as regulators in metabolic processes, as messengers and as antibiotics and chemotherapeutic agents.

This review will be limited to a study of the analysis by high performance liquid chromatography (HPLC) of nucleosides and bases. Other low molecular weight, UV-absorbing compounds which are often found with the nucleosides in various physiological fluids will also be discussed. HPLC has revolutionized the analysis of compounds such as the nucleosides in complex biological fluids or extracts. The efficiency and the sensitivity of the latest HPLC techniques are immeasurably better than the earliest LC work, and the time of analysis has decreased dramatically.

It was felt that a brief discussion of the nomenclature of the nucleosides and bases would provide a convenient and helpful source of material on this sometimes confusing subject. Following this, a comprehensive review of the chromatographic methods of

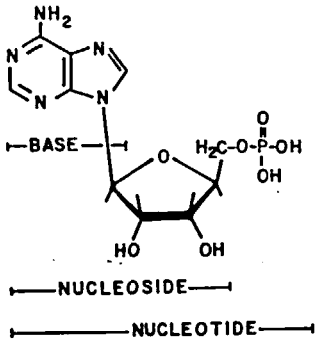

FIGURE 1. Adenosine monophosphate (AMP) with the three levels of molecular structure from which it is derived.

analysis available for these compounds will be presented. The sample preparation techniques, which are of fundamental significance to the accurate analysis of the nucleic acid components in body fluids and tissues, will also be discussed. After this, a discussion of the various identification techniques useful for the nucleosides and bases will be presented. The last segment of the review will be concerned with some of the applications of these techniques in biomedical studies.

B. Nomenclature of the Nucleic Acid Components

A complete review of systematic nomenclature rules for heterocyclic compounds is beyond the scope and purpose of this review. Nevertheless, because heterocyclic nomenclature can be quite complex for larger ring systems, a short introduction to those rules will be made.

Many of the heterocyclic compounds encountered in biochemical work have at least two names, a biochemical and a systematic chemical name. To further complicate matters, a trivial name is often given to frequently used compounds.

Starting with the heterocycle itself, which is called the base or the aglycone, higher order structures are constructed by the addition of integral molecules, such as ribosides, phosphate groups, etc. Figure 1 shows these levels of structures for one of the purines, adenine. Adenine is the common name for the purine base shown in Figure 1. When a base is combined with a sugar group, the molecule is referred to as a nucleoside. If the molecule contains a base, a sugar moiety, and one or more phosphate groups, it is referred to as a nucleotide. Nucleotides can be much more complex than the simplest 5′-mononucleotide shown in Figure 1. For example, nicotinamide adenine dinucleotide (NAD) is composed of two distinct aglycones, a ribosyl sugar and two phosphate groups.

1. Naming the Aglycones

Before giving systematic names to the more complex structures of the nucleosides, nucleotides, or polynucleotides, it is necessary to name the aglycone ring system itself. Recent efforts by the International Union of Pure and Applied Chemistry (IUPAC) have been directed towards the systemization of the nomenclature of the diverse area of heterocycles.[1] In cases where the historical precedent could not be overcome, the common names were accepted as official.

In naming a heterocycle, there are two problems. First, the ring system itself must be named. Then the entire aglycone with its substituent groups must be named. Table

Table 1
NOMENCLATURE OF HETEROCYCLES

A. Prefixes Used for the Naming of Heterocycles

Element	Valence	Prefix
Oxygen	2	Oxa
Sulfur	2	Thia
Nitrogen	3	Aza
Silicon	4	Sila

B. Suffixes Used to Indicate Ring Size and Saturation

No. of ring members	Nitrogen-containing rings		Rings with no nitrogen	
	Unsaturated	Saturated	Unsaturated	Saturated
3	-irine	-iridine	-irene	-irane
4	-ete	-etidine	-ete	-etane
5	-ole	-olidine	-ole	-olane
6	-ine	**	-ine	-ane
7	-epine	**	-epine	-epane

* Expressed by using the prefix "perhydro" with the name.

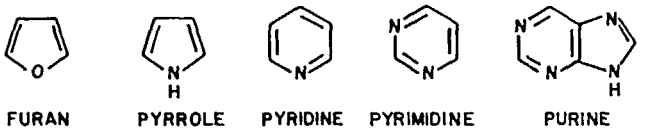

FIGURE 2. Molecular structures of several of the commonly encountered heterocycles with their common names.

1 (A and B) outlines the rules of nomenclature for the ring system. The ring is named by adding a prefix according to the heteroatom contained therein. A nitrogenous ring would be an aza compound, a sulfur-containing ring a thia compound, etc. If two heteroatoms are contained, a double prefix is given, with the higher atomic number element being named first.

Both the size of the ring and its degree of saturation are indicated by a suffix. As Table 1 (A and B) shows a five-member unsaturated ring system containing a nitrogen atom would belong to the azole family, a seven-member unsaturated oxygen ring would be called oxepin, etc.

Those ring systems which were widely known before the systematizing of the nomenclature often have retained their common names as official ones. Therefore, the structures shown in Figure 2 are official, but not systematic names. Furan, pyrrole, pyridine, pyrimidine, and purine are examples which are commonly encountered.

When two or more rings are fused together, as for example purine in Figure 2, it is necessary to designate the positions of the heteroatoms within the ring. Figure 3 shows a ring system in which this is done. The first step is to number the smaller ring internally so that the shortest route (the lowest total numbers of the heteroatoms) is found through the bridgehead atoms, which includes all the heteroatoms within the smaller ring. The numbering is begun with one of the heteroatoms. In Figure 3, the pyrrolo

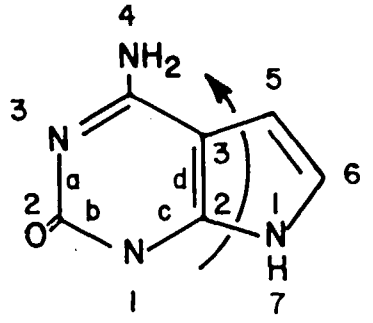

FIGURE 3. An example of the systematic naming of the heterocycle 4-amino-pyrrolo-(2,3-d)pyrimidin-2-one. The ring system itself is named by the combination of the bridgehead numbers and the common bond letter of the larger ring with the names of the smaller and larger rings. The numbers around the outside of the ring are arranged so as to produce the lowest positional sum of the ring heteroatoms.

ring is numbered with the pyrrolo nitrogen as locant number 1, with the two bridgehead atoms as 2 and 3.

Next, the bonds of the larger ring are alphabetically lettered beginning with one of the heteroatom bonds and progressing in the direction which will give the shortest route to the bond connecting the bridgehead atoms. Thus, the common bond in Figure 3 is lettered as d (always a small letter). The direction of the bond lettering within the larger ring is noted (clockwise or anticlockwise). The members of the two bridgehead atoms as given by the smaller ring are listed in the order encountered by the clockwise or anticlockwise direction of the large ring letters. The combined ring system is named with the smaller ring first then, in parentheses, the two bridgehead atoms locant numbers, a dash, and then the letter of the bridgehead bond. After the parenthesis, the ring name of the larger ring is given. Therefore, the basic heterocyclic system of Figure 3 would be a pyrrolo(2,3-d)pyrimidine. The basic purine ring system which will be frequently encountered would be systematically named as an imidazolo(4,5-d)pyrimidine. However, purine is an exception to the rules of nomenclature and is almost always referred to by its common name, either purine or some purine derivative.

Once the ring system itself is determined, it is necessary to number the substituent groups. For any ring system except purine, the numbering system is similar to the carbocyclic nomenclature and is done in such a way as to minimize the sum of the positional or locant numbers of the heteroatoms. The number 1 position of the bicyclic ring systems must begin α to one of the bridgehead atoms. For monocyclic systems, one of the heteroatoms is numbered as 1, with the numbering progressing around the ring so as to yield the lowest total for the heteroatom positions. It is important to note that the substituent groups themselves do not determine the positional number assigned to them. Thus, the compound of Figure 3 would be named 4-amino-pyrrolo(2,3-

FIGURE 4. The ring numbering of the purine and pyrimidine ring systems and the D-ribofuranosyl group. The purine ring numbering is not in accordance with the systematic rules of nomenclature due to the large historical precedent associated with the chemistry of the purine ring. In the ribofuranosyl group, the 1′ position is anomeric. Most of the nucleosides commonly encountered in mammalian systems will be the β configuration (1′ and 4′ bonds syn to each other).

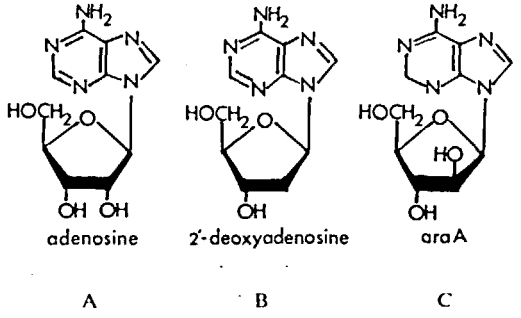

FIGURE 5. Three different nucleosides with different sugar groups but with the same aglycone moiety. The three sugar groups are (A) the β-D-ribofuranosyl (adenosine), (B) the β-D-erythropentofuranosyl (2′-deoxyadenosine) and (C) the β-D-arabinofuranosyl (ara-A) groups.

d)pyrimidin-2-one, and the substituent groups are listed alphabetically in the molecular name.

An inconsistency arises with the purine ring system. Because of the historical precedence of the chemistry of this biologically ubiquitous structure, it is not numbered in accordance with the systematic method just outlined. Purine-derived compounds are numbered according to Figure 4. The pyrimidine ring is also shown in Figure 4. Since it is monocyclic, its numbering and nomenclature is simpler and unambiguous.

2. Naming the Sugars

Since a sugar moiety is always present in a nucleoside or nucleotide, the sugars present must be unambiguously identified.[2] Carbohydrate nomenclature can become even more complex than that of the aglycone itself. Fortunately, only a few of the more common sugar residues will be encountered in routine work. Figure 5 shows three commonly encountered structures, the ribofuranosyl, the deoxyribofuranosyl, and arabinofuranosyl groups.

Whenever a sugar is named as a substituent group rather than a distinct molecule, it is suffixed with -yl rather than -ide. The structure shown in Figure 5A is the D-ribofuranosyl group. The ribose indicates a specific pentose sugar, while the furan indicates a cyclic furanose-like ring. When the sugar is a substituent group, all sugar

Table 2
SUMMARY OF USEFUL PHYSICAL AND SPECTROSCOPIC DATA FOR SOME COMMONLY ENCOUNTERED PURINE AND PYRIMIDINE NUCLEOSIDES AND BASES

Name	Mol wt	pK_a	pK_b	λ max (nm) In acid soln	In neutral soln	In alkaline soln
Uracil	114.10	9.45	—	—	—	230
Uridine	244.20	9.17,12.5	—	262	262	265
Cytosine	111.10	12.2	4.45	276	267	282
Cytidine	243.22	12.5	4.15	280	230,271	273
Thymine	126.11	9.9	—	265	265	291
d-Thymidine*	242.23	9.8	—	267	267	267
5-Methylcytosine	125.13	12.4	4.6	283	273	289
5-Methylcytidine	257.24	13	4.3	287	277	279
Adenine	135.13	9.8	1,4.15	263	261	269
Adenosine	267.24	12.5	3.5	257	260	259
Guanine	151.13	9.6,12.4	3.2	248,276	246,276	274
Guanosine	283.24	9.2,12.4	1.6	256	253	256,266
Hypoxanthine	136.11	8.9,12.1	2.0	248	250	263
Inosine	268.23	8.8,12.3	1.2	248	249	253
1-Methylhypoxanthine	150.14	8.9,13	2	249	251	260
1-Methylinosine	282.25	—	—	250	251	249
Xanthine	152.11	7.5,11.1	0.8	260	267	277
Xanthosine	284.23	5.7	2.5	235,263	248,278	252,276

* The base thymine is found as the deoxynucleoside in most biological systems.

positions are numbered with primes so that the anomeric position is 1′. Whether a pentofuranosyl sugar is D or L is determined by the 5′ position. Thus, if the 9 position of the adenine aglycone were joined to the 1′ position sugar of Figure 5A (with the glycosyl bond being "up"), the resulting molecule would be named 6-amino-9-β-D-ribofuranosyl-purine, otherwise known as adenosine.

The deoxy sugars are also quite common (Figure 5B). While structure 5B is often referred to as the 2′-deoxy- , it is formally a D-erythropentofuranosyl structure. Various other sugars are commonly encountered when dealing with microbial biology, such as the D-arabinofuranosyl- group shown in Figure 5. When combined to an adenine aglycone at the 9 position of the base, the resultant molecule is named 6-amino-9-β-D-arabinofuranosyl-purine, which is given the common name of ara-A. Ara-A is one of the newest anticancer drugs which appears to be effective against certain types of leukemias.[2]

Table 2 summarizes the nomenclature and physical data for some commonly encountered nucleosides and bases, based upon the purine and pyrimidine ring systems. The nucleotides are not listed, but their nomenclature is derived from the nucleoside in a straightforward manner.

3. Tautomeric Forms

Considerable confusion in the earlier literature arose concerning the tautomeric forms of several important substituent groups. Figure 6 shows the lactam-amino form (A) and the lactim-imino form (B) of the purine base guanine. It has been demonstrated both theoretically and experimentally[4] that the lactim-amino tautomers are usually the thermodynamically favored structures in purines and pyrimidines under physiological conditions.

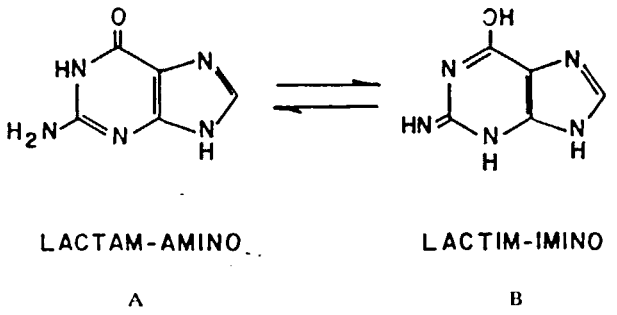

FIGURE 6. Two of several possible tautomeric structures of the base guanine. The lactam-amino structure is the predominant species under physiological conditions.

C. Physical Properties

In the analysis of the nucleosides and bases, it is often essential to know the pKa and pKb values of the molecule along with the spectral properties (λ_{max} and molar absorptivity coefficient) in a given solvent at several pH values (Table 2). Identification of a chromatographically pure nucleoside, base, or nucleotide is sometimes possible from the UV absorption characteristics alone. It should be noted that in many instances the UV absorption curves of nucleosides will be qualitatively similar. *The Merck Index*[5] and the *Handbook of Biochemistry*[6] are two good sources of information on these compounds.

D. Biochemical Significance of Nucleosides and Bases

Nucleosides and bases are the basis for the nucleotides. The nitrogenous compounds of the body are formed either *de novo* or are recycled through the purine salvage pathway. The bases and nucleosides are of central importance to numerous areas of research in DNA-RNA synthesis and in the basic metabolic processes.

An area of research which has generated considerable interest in recent years has been that involving the modified nucleosides. These compounds were once thought to be anomalies of the isolation process of RNA, but are now known to be of fundamental significance to the proper functioning of t-RNA and other genetic material.

More than 40 different methylated (modified) nucleosides and bases have been isolated from t-RNA of many species, including man.[7] Modified base structures have also been shown to exist in the messenger and ribosomal RNAs. However, these nucleic acids are difficult to work with in the laboratory and thus less is known about the role of the methylated compounds. Likewise, some DNA polymers have been demonstrated to contain 5-methylcytidine, the only modified nucleoside found to date in mammalian cells.[8-10]

All alkylations of nucleic acid materials are believed to occur within the polynucleotide chain. Modified nucleosides are not reincorporated into the nucleic acid material, but are released into the blood and are excreted in the urine.[11,12] The only demonstrated role of the modified nucleosides in DNA is that of host recognition. Nuclease enzymes have been shown to recognize foreign DNA material and degrade it, while not reacting with host DNA with specific methylation patterns.[13,14]

It is well known that certain alkylating compounds are highly carcinogenic. Diverse groups of compounds, such as epoxides, lactones, alkyl sulfates and halides, as well as the potent alkylating agents, the nitrosamines, are all cancer-causing agents. Conversely, the alkylating agents are also used in anticancer therapy as cross-linking agents for the rapidly dividing tumor DNA.[15]

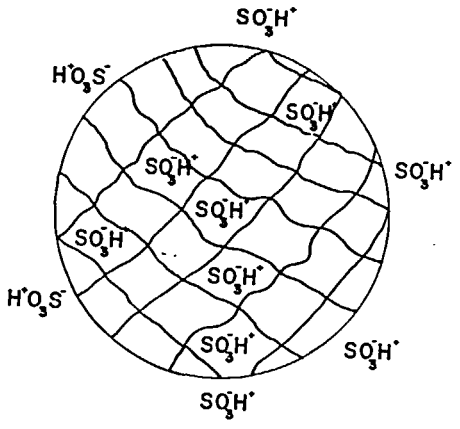

FIGURE 7. A schematic diagram of structure of a polystyrene-divinylbenzene type of cation-exchange resin.

The presence of certain of the methylated nucleosides and bases in the urine of cancer patients has sparked considerable interest in the area of biochemistry. HPLC and other techniques have made possible sensitive identification of compounds in complex matrices such as urine and blood. The most recent discoveries in this area of research will be discussed in Section V.

II. CHROMATOGRAPHY OF THE NUCLEOSIDES AND BASES

The advances in the efficiency and selectivity of HPLC separations of the nucleosides and similar compounds have been the result of extensive development of the theories of band broadening and the chemistry of the separation process itself. The basic theory of HPLC, while still under active development, has become a part of the chemical literature. Several excellent monographs have appeared in the last several years[16-23] giving in-depth reviews of the current status of the latest theoretical advances. There are two generalized aspects of theory: one deals with the efficiencies of column and particle designs, which is generally classified within the area of kinetics and the other deals with the actual retention times (k′ values) as a function of the thermodynamic properties of the chromatographic system. Considerable research on the kinetic aspects have yielded tremendous advances in column efficiencies. More work is needed to understand the thermodynamics of the separation of compounds by HPLC. An increased understanding will lead to better control and prediction of the retention times.

A. Ion Exchange

Cohn's work at Oak Ridge in the early 1950s[24] utilized polystyrenedivinyl benzene ion exchangers for the separation of the constituents of nucleic acids. The use of these resins in low-pressure and open-column work revolutionized the analysis of nucleotides, nucleosides, and bases. The nucleosides and bases, many of which have acidic or basic moieties, have until recently been most often separated by ion exchange.

The earliest resins used for chromatographic work had particle diameters on the order of 70 μm and were totally porous. Figure 7 shows a schematic diagram of the structure of a cation exchange resin bead. The chemistry and applications of such ion exchangers has been detailed in several reviews.[25,26]

These resins proved to be useful for the separation of microgram-milligram quantities of the nucleic acid components using the slower flow rates of the open-column separations. However, problems arose when such resins were used in higher-performance chromatographic systems. A brief discussion of some of the factors contributing to band broadening will show why the large particle resins were incompatible with the higher flow velocities of modern HPLC.

The object of a high-efficiency chromatographic column is to minimize the dispersion of the solute band as it traverses the length of the column. The degree of this dispersion is characterized by the plate height H, where H is measured on the chromatogram according to Equation 1:

$$H = L/N = \frac{L}{16}\left(\frac{Wb}{tr}\right)^2 \quad (1)$$

where L is the column length, W_b is the width of the peak at its base and t_r is the retention time of the peak. All measurements are in consistent units.

Knox has found[17] that it is often more meaningful to express column efficiencies in terms of reduced plate height h,

$$h = H/d_p \quad (2)$$

which is essentially the plate height measured in particle diameters (d_p). The reduced plate height was first pioneered by Giddings.[19]

It should be noted that many of the equations developed to describe the chromatographic behavior of a solute band, such as plate number N or retention k', are applicable only under isocratic conditions. Gradient elution, where the composition of the mobile phase is continuously varied, prevents the use of any functions which assume constant solvent composition. Thus terms such as plate height H or separation factor α, while measurable on a gradient elution chromatogram, lose their fundamental significance in such a system.

Using the reduced plate height terminology, the total plate height (or variance) produced by a chromatographic column is the sum of the individual contributions to that height by a variety of factors. In a form similar to the Van Deemter equation, there are at least three major contributions to plate height. These are longitudinal diffusion (h_{diff}), eddy diffusion (h_{flow}), and resistance to mass transfer (nonequilibration, h_{ne}). Thus the total plate height produced by a column may be approximated as

$$h = h_{diff} + h_{flow} + h_{ne} \quad (3)$$

In an expanded form, Equation 3 becomes

$$h = \frac{B}{\nu} + A\nu + C\nu \quad (4)$$

where B, A, and C are each complex terms describing the system.

ν is a special term referred to as the reduced velocity

$$\nu = \frac{\mu d_p}{D_m} \quad (5)$$

μ is the flow velocity, and D_m is the diffusion coefficient in the mobile phase.

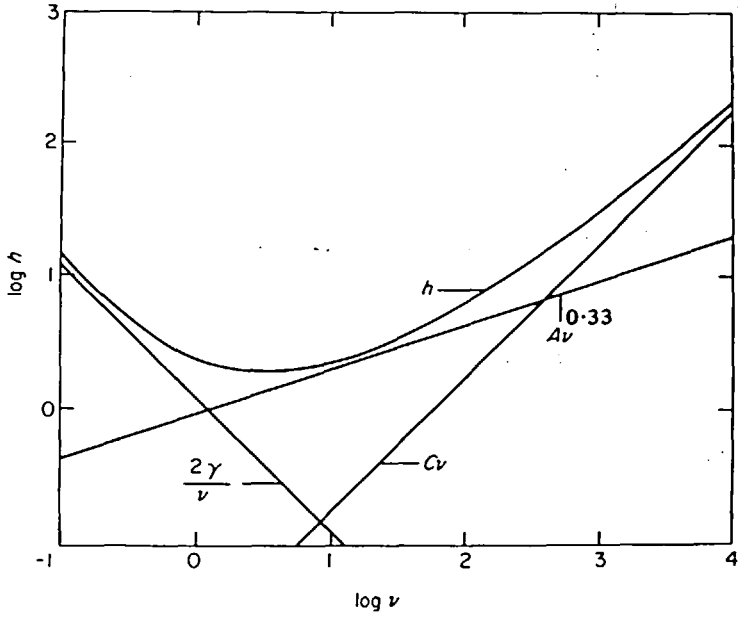

FIGURE 8. Logrithmic Van Deemter-type plot of the reduced plate height h against the reduced velocity of the mobile phase ν showing typical contributions to the overall plate height from the eddy diffusion (A ν), longitudinal diffusion ($2\gamma/\nu$), and resistance to mass transfer terms (C ν).

Figure 8 shows the general form of Equation 4. The primary advantage of using the reduced parameter approach of Knox is that valid comparison between the true efficiencies of various packing materials can be made despite differences in particle size.

The portion of Equation 4 which is of interest to the discussion concerning ion-exchange resins is the term h_{ne}. In general, the h_{ne} term involves the time required for a solute molecule to diffuse from the flowing mobile phase into the pores of the support to the active sites, exchange, and then to return to the mobile phase. The h_{ne} term is of such a nature that h_{ne} is directly proportional to the particle diameter and inversely proportional to the diffusion coefficient D, of the mobile phase entrapped within the particle.

Because of the large h_{ne} term, the first conventional polystyrene ion exchange resins gave generally poor efficiency. Improvements of the polymeric resins involved reducing the particle diameter to the 5 to 10 μm range and determining the degree of cross-linking needed to give the best balance between particle rigidity and rapid diffusion into and out of the pores. These improvements have enabled the polymeric resins to be used in modern HPLC systems.[25]

The nucleosides and bases were separated routinely on both the anion[27,28] and cation[39-47] exchange resins. Figure 9 shows the separation of many of the biochemically important nucleosides and bases on an anion-exchange resin at pH 9.7.[32] The greatest advantage of these resins over more recently developed packing materials is their high capacity (several milliequivalents per gram), low cost, and ease of packing. Although the newer microparticulate, chemically bonded resins offer higher efficiencies and are mechanically more rigid, the polymeric resins are still useful media for the separation of a wide variety of compounds. Floridi et al.[35] were able to separate many of the bases, nucleosides, and nucleotides on a single anion-exchange column. However a separation time of 225 min was required, which illustrates the mass-transfer limiting nature of the resins and the necessity to work at relatively low flow velocities.

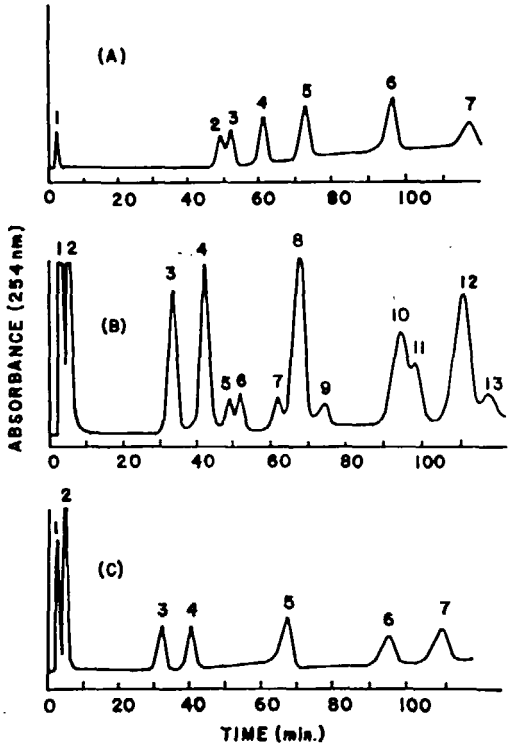

FIGURE 9. Separation of purine and pyrimidine bases and nucleosides by HPLC using a conventional type strong anion-exchange resin. (A) Separation of purine and pyrimidine bases, (B) separation of the purine and pyrimidine nucleosides and bases, and (C) separation or purine and pyrimidine nucleosides. Contained under each peak are 25 to 75 nmol. The chromatographic conditions were as follows. Effluents: low concentration, 0.05 mol/*l* ammonium acetate, pH 9.7; high concentration, 1.00 mol/*l* ammonium acetate, pH 9.7. Gradient: using a mixing chamber device, initial volume, 50 m*l*, 6 m*l*/hr gradient flow rate. Column flow rate: 12 m*l*/hr. Temperature: 70°C. Column packing material: Aminex® A-28. (Reproduced from Brown, P. R., *J. Chromatogr.*, 99, 587 (1974). With permission.)

In an effort to overcome several of the inherent limitations of the polymeric, totally porous resins, new types of particles called the pellicular resins or superficially porous packings were developed and applied to nucleoside and base separations by Horvath and Lipsky,[48] Kirkland,[49] and others[50,51] in the late 1960s. In an effort to reduce the diffusion distance of the solute molecules into the stationary phase, the ion-exchange resin was polymerized around a solid glass or silica support particle, usually between 40 to 70 μm in diameter. When properly manufactured, such packing materials offer substantial increases in efficiency over the polymeric resins and were successfully used for the separation of the nucleosides and bases.[48]

Figure 10 shows a plot of the log of the reduced plate height against the log of the reduced velocity of the mobile phase. Although the conventional ion exchangers show a higher efficiency at very low mobile phase velocities, the higher efficiency of the superficially porous or pellicular ion exchangers at higher flow rates is evident. In

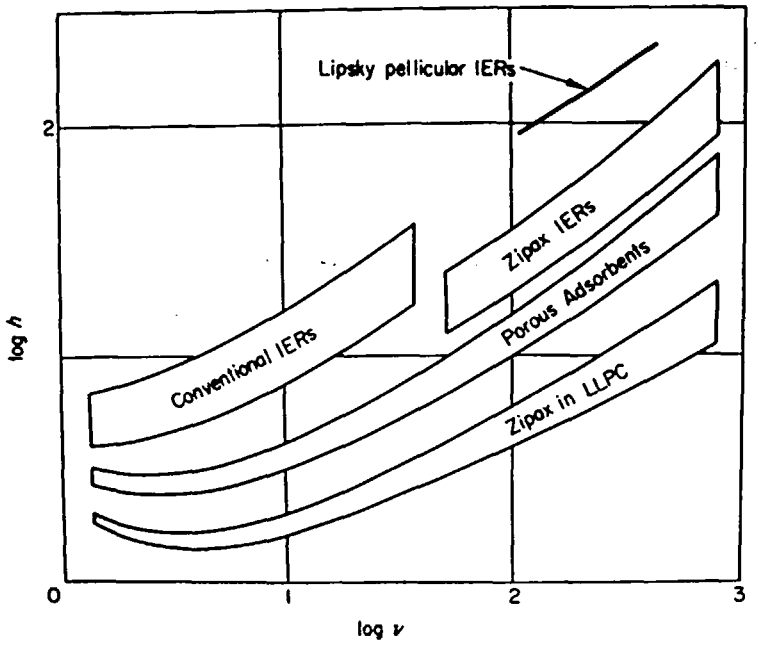

FIGURE 10. Reduced plate height curves for various types of packing materials showing the relatively poor performance of ion-exchange resin materials.

general, the use of the pellicular design offered improvements over the conventional resins in terms of better efficiencies at higher flow rates, thus reducing the time of analysis. The increased mechanical rigidity enabled the pellicular material to withstand the higher mobile phase velocities.

The pellicular resins, though successful in increasing the efficiency of the HPLC column, suffered from two serious limitations. First, because of the design of the particle itself, the ion-exchange capacities of the pellicular packings were extremely low, usually on the order of only several microequivalents per gram. The second problem was that of stability. The earliest pellicular packings tended to shed their polymeric "skin", resulting in peak trailing and unstable retention times due to exposed support material.

The latter problem was successfully resolved by the introduction of chemically bonded packing materials which are superficially porous. The technology which was developed paved the way for the introduction of the chemically bonded microparticulate particles which are in widespread use today. In the superficially porous chemically bonded packings, the active sites were chemically bonded to a microetched glass or silica support with a diameter of 30 to 70 μm. These packings proved to be chemically and mechanically more stable than the first pellicular packings and were successfully used to separate the nucleosides and bases[49] as well as a wide variety of other compounds. However, the problem of extremely low sample capacity remained, usually limiting the sample size to the picomole-nanomole range.

B. Ion Exclusion

A novel application of the ion-exchange process was reported by Singhal and Cohn in a series of papers describing the ion-exclusion chromatography of the nucleosides and bases.[52-55] In this process, the "wrong" pH is used so that the solute molecules carry a positive charge for anion-exchange columns, and a negative charge for cation-

exchange columns. Those molecules which have a greater charge will be excluded from the support pores to a greater degree than lesser-charged species and will consequently elute sooner. While useful in certain instances, ion-exclusion chromatographic separations have lacked the flexibility found in conventional ion-exchange separations.

C. Gel Packings and Miscellaneous Techniques

Systems other than ion exchange were utilized for the separation of the nucleosides and bases. DEAE cellulose (which functions as an anion exchanger), polyacrylamide, and other gel matrices[56-67] have been quite widely used.

Both ligand-exchange and affinity packing materials have been successfully used for the selective retention of several of the nucleosides and bases.[68,69] The affinity columns are based on the same selective hydrogen bonding characteristics of the base pairs that occurs in the DNA chains. Most of these separations, however, have not offered general improvements in the way of column efficiency or selectivity. Nevertheless, such techniques remain useful in sample preparation or prepurification and group separations.

D. Chemically Bonded Microparticulate Packings

1. Ion Exchange

The next development in the design of higher efficiency packing materials for HPLC came in the form of ion exchange or other groups being chemically bonded to small (10 μm) totally porous silica supports. The smaller particle diameters and the porosity of the support tremendously increased the surface area, resulting in much greater exchange capacities than with the pelliculars. Moreover, column efficiency was high because of the limited amount of stagnant mobile phase held up within the small particles.

Small particle anion-exchange packings proved to be highly suitable for the separation of nucleotides,[70] which carry one or more ionized phosphate groups at pH values above 3 to 4. The high efficiency of these packings produced very narrow solute bands, thus increasing the sensitivity of the analysis well into the picomole range for compounds with good detection properties. The higher capacity simultaneously increased column capacities into the microgram range.

In the chemically bonded particles, the chemical groups are attached to the silica via a simple chlorosilane-silica gel reaction as shown in Figure 11. The chemistry and chromatography of the bonded phases has been the subject of an excellent review by Grushka and Kikta[71] as well as a comprehensive text.[72] In ion-exchange packings, the R groups shown in Figure 11 are usually long-chain alkyl groups which contain quaternary amine (strong anion exchange) or a sulfonic acid (strong cation exchange) moieties at the end of the molecule. Thus the ion-exchange groups are chemically bonded to the silica via "spacer" molecules. In addition to the strong ion exchangers mentioned, a wide variety of weak anion- and cation-exchange packings are also available.

Figure 12 shows a chromatogram of seven nucleosides and a chromatogram of seven bases on a microparticulate, chemically bonded ion-exchange resin. In addition to the fact that the separation of the nucleosides is poor, a pH of 2.8 was found to produce the best separation on the cation-exchange packing. This represents the minimum pH possible before the silane bond becomes susceptible to hydrolysis. Many of the nucleosides and bases are ionized at high pH values, thus anion exchange has been the more widely used ion-exchange mode for these compounds. However, in order to ionize many of the purine and pyrimidine compounds, a pH of 8 to 10 is usually required. Since acidic silica gel support is susceptible to dissolution at high pH, the upper limit of eluent pH is usually considered to be 7.0 to 7.5. Thus, silica-based packings are

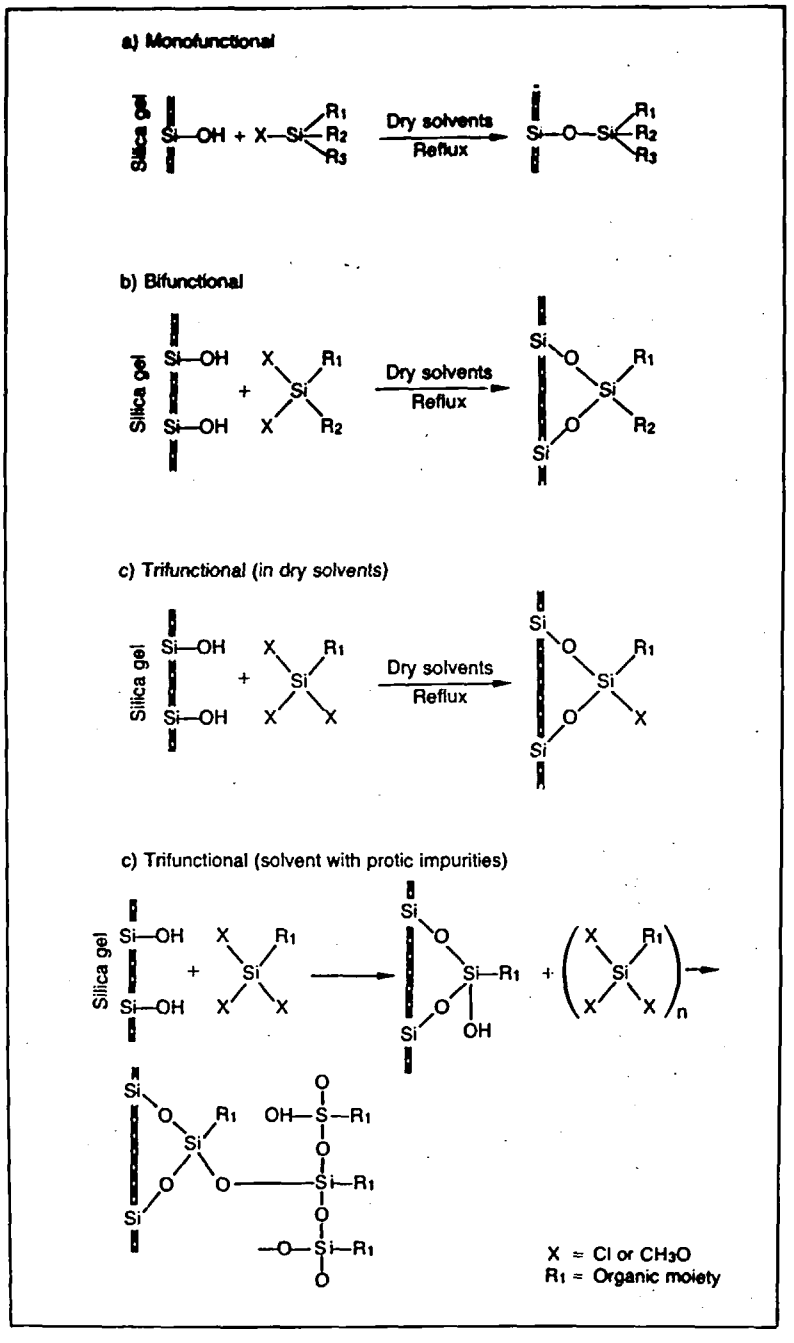

FIGURE 11. Schematic of the reaction of the chlor- or alkoxysilanes and silica gel for the formation of various bonded stationary phases. (Reprinted with permission from Grushka, E. and Kikta, E. J., *Anal. Chem.*, 49(12), 1004A, (1977). Copyright by the American Chemical Society.)

incompatible with the anion-exchange separation of many of the nucleosides and bases.

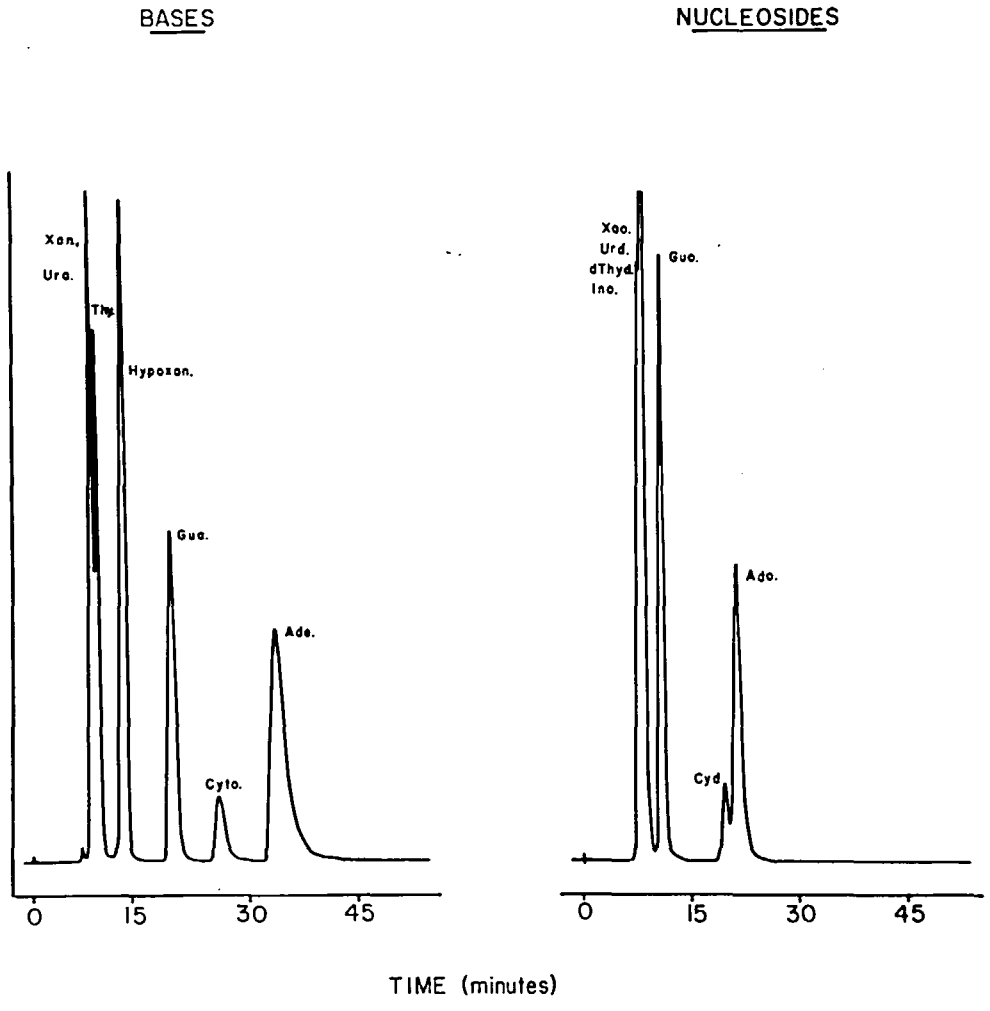

FIGURE 12. Separation of several of the biologically important nucleosides and bases using chemically bonded strong cation-exchange microparticulate packing material. Chromatographic conditions: eluent: 0.005 mol/*l* KH_2PO, pH 2.8, flow rate: 1.0 m*l*/min, temperature: ambient. Detection at 254 nm.

2. Reversed Phase

The introduction of the reversed-phase chemically bonded pacings in HPLC has revolutionized the separation of the nucleosides, bases,[73-79] and other nonionic compounds. Reversed-phase packings proved to be highly suitable for the separation of compounds with a wide range of polarity. The microparticulate reversed-phase materials are of the same design as the chemically bonded ion exchangers discussed above except that no ion-exchange moiety is present at the end of the organic chain.

The exact mechanism of solute retention on reversed-phase packing materials is not presently well understood and remains the subject of active research.[80-86] Whatever the exact mechanism is found to be, it is fairly evident that it is not a simple partition process by which solute molecules are retained in (or on) the stationary phase. In many respects, the bonded organic groups act as chemical modifiers of the silica surface, with the mobile phase itself probably playing an active role in determining the surface activity.

Regardless of the mechanism, the reversed-phase packing materials have proven to

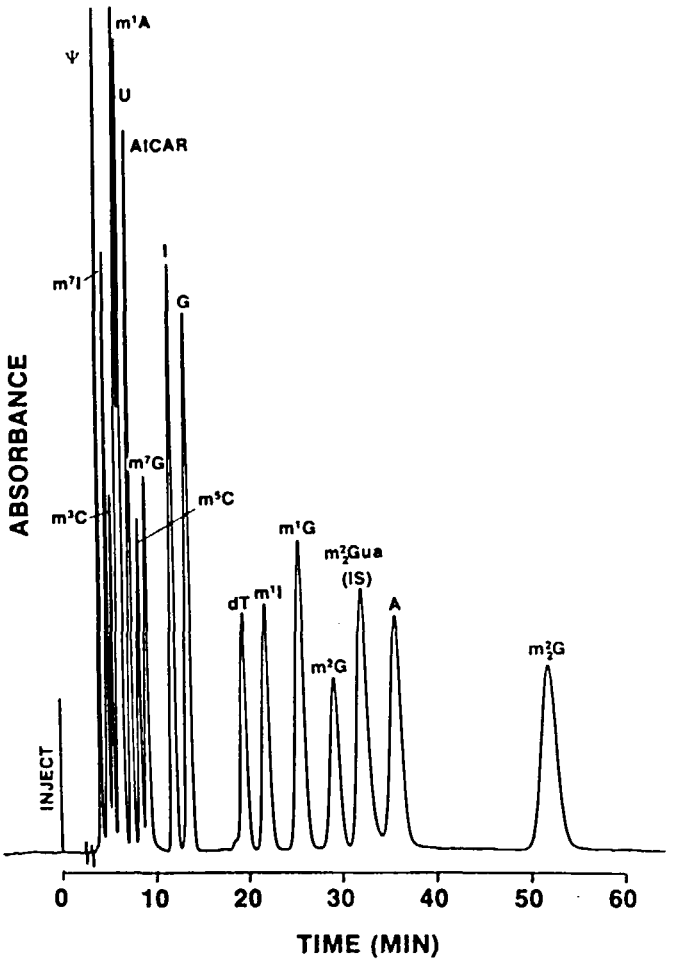

FIGURE 13. Reversed-phase HPLC isocratic separation of a mixture of 16 nucleosides. Sample size: 500 pmol of each nucleoside. Column. Microbondapak® C_{18} (4 × 300 mm); mobile phase: 0.01 mol/*l* $NH_4H_2PO_4$, pH 5.07, with 60 m*l* of methanol added per liter; flow rate: 1.0 m*l*/min; detection at 254 nm; temperature: ambient. (Reproduced from Davis, G. E., Suits, R. D., Kuo, K. C., Gehrke, C. U., Waalkes, T. P., and Borek, E., *Clin. Chem. (Winston-Salem).*

be highly suitable for the separation of the nucleosides and bases. At intermediate pH values from 3 to 7, most of the nucleosides and bases do not carry formal charges. This renders the ion-exchange mode to be ineffective within this pH range, but makes the reversed-phase mode of HPLC ideally suited.

Figure 13 shows the separation of 16 of the ribonucleosides using 10-μm C_{18} packing material using isocratic elution. The mobile phase consists of a mixture of 60 m*l* of methanol in 1 *l* of 0.01 mol/*l* $NH_4H_2PO_4$, pH 5.07. Excellent resolution of the compounds is achieved in about 55 min at a flow rate of 1.0 m*l*/min. The reversed-phase columns usually function well at room temperature, unlike the ion-exchange resins which are often more efficient at elevated temperatures.

a. Effect of Organic Modifier

When using the reversed-phase mode of HPLC, the most polar solutes are eluted

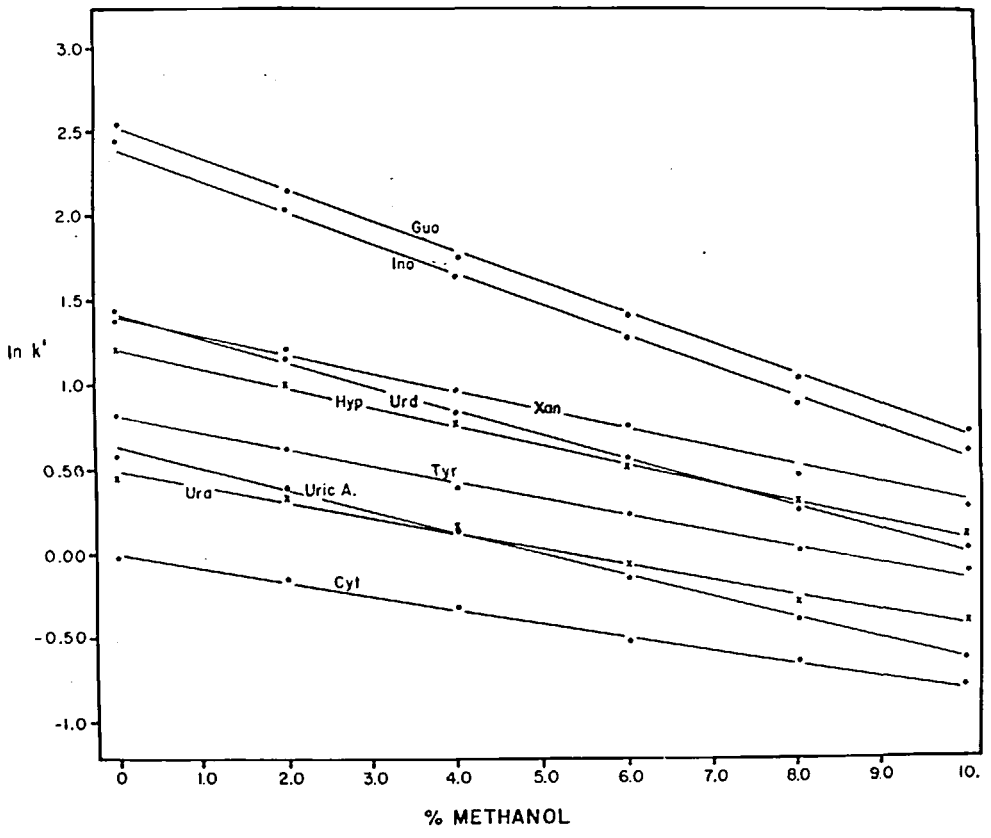

FIGURE 14. The effect of methanol concentration in the mobile phase. Ln k' of the nucleosides and other compounds versus % methanol. Column: Partisil®-5 ODS (C_{18}); temperature: 25°C; flow rate: 1.0 m*l*/min. (Reprinted with permission from Hartwick, R. A., Grill, C. M., and Brown, P. R., *Anal. Chem.*, 51, 34 (1979). Copyright by American Chemical Society.)

first, while lipophilic compounds generally are the most strongly retained. Conversely, the most polar mobile phase, such as water, is the weakest eluent in the reversed-phase system. For the nucleosides and bases, methanol or acetonitrile are the organic modifiers often used to adjust the retention times. Figure 14 shows the effect of methanol concentration in the mobile phase on the separation of six of the nucleosides, bases, and aromatic amino acids. The relationship between k' and % organic solvent appears to be logrithmic[83] for reversed phase, at least over a limited range of organic solvent concentrations. For high concentrations of organic solvent, the logrithmic relationship is not always linear and a higher order equation must be used to describe the system.

b. Effect of pH

Figure 15 shows the effect of the pH of the mobile phase on the retention time of various nucleosides and bases on a reversed-phase (C_{18}) column. The retention volumes of the solutes remain constant except when a solute becomes ionized. Thus, the nucleoside xanthosine (pKa 5.7) undergoes a change in retention time that approximates the sigmoidal shape of a titration curve as the eluent pH is varied through this region. Such pH-dependent changes can be extremely useful in developing separations selective for individual solutes or classes of compounds.

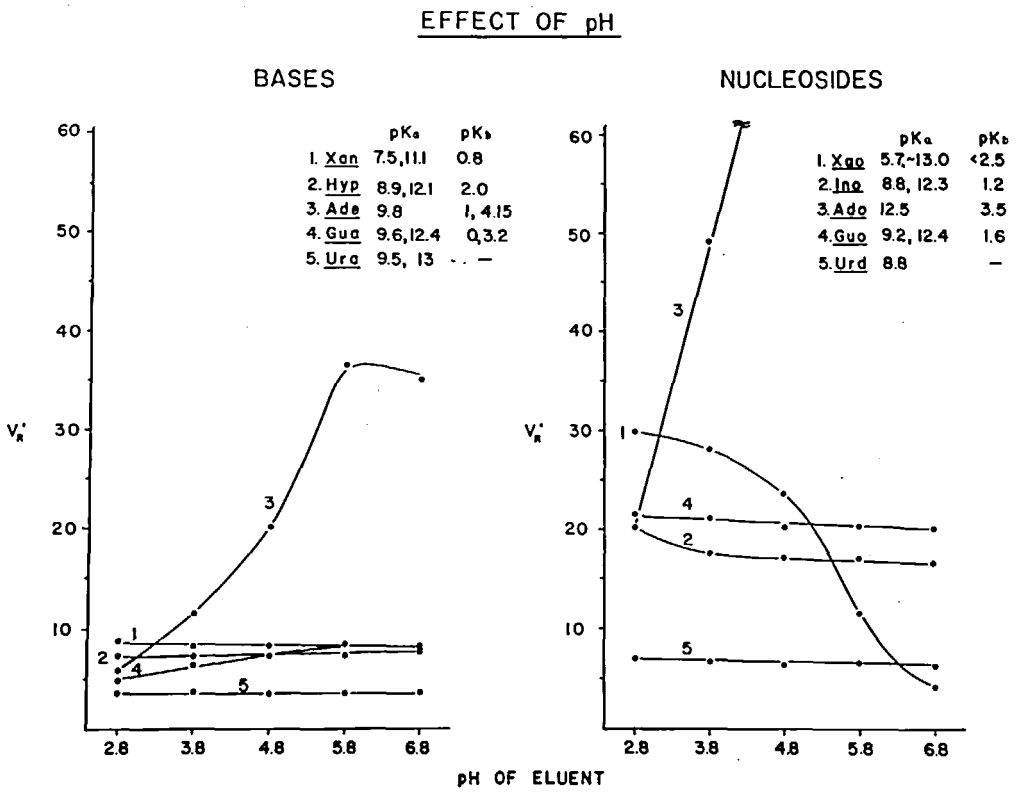

FIGURE 15. Effect of pH of the mobile phase on the retention volumes of the nucleosides and bases on a reversed-phase, C_{18} column. The ionic strength of the mobile phase was held approximately constant by the addition of 0.1 mol/*l* KCL in a 0.01 mol/*l* KH_2PO_4 buffer. Column: Microbondapak® C_{18}; flow: 1.5 m*l*/min; temperature: ambient. Reprinted with permission from Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 126, 679 (1976). With permission.)

c. Gradient Elution

Gradient separations on reversed phase are possible, which allow for the detection and quantitation of picogram quantities of not only the nucleosides, but also the bases and other low molecular weight, UV-absorbing compounds as well. The use of gradient elution extends the range of compounds which can be separated within a given time frame by continuously increasing the solvent strength of the eluent with time, thus decreasing the effective k' of those solutes which would otherwise elute with unreasonably long retention times. The separation of 29 of the nucleosides, bases, aromatic amino acids, and several nucleotides is shown in Figure 16. The high efficiency of the columns and strong absorption of the purine and pyrimidine compounds in the UV range allow for sensitive and accurate quantitation of these compounds, with the lower limit of detection for most of the nucleosides being about 50 pmol.[74,77]

For those solute molecules which are fully ionized at the working pH of the separation, the technique of ion pairing can be of great utility in extending the usefulness of the reversed-phase column.[87-92] In this technique, a counter ion is added to the mobile phase. A complex series of equilibria are thus set up between the solute-counter ion, the counter ion-stationary phase, and the solute-counter ion-stationary phase. The ionic charge of the solute ion is effectively neutralized by the counter ion, and the resulting complex is retained on the nonpolar stationary phase. The nucleotides have recently been separated on a reversed-phase column using tetra-*N*-butylammonium as

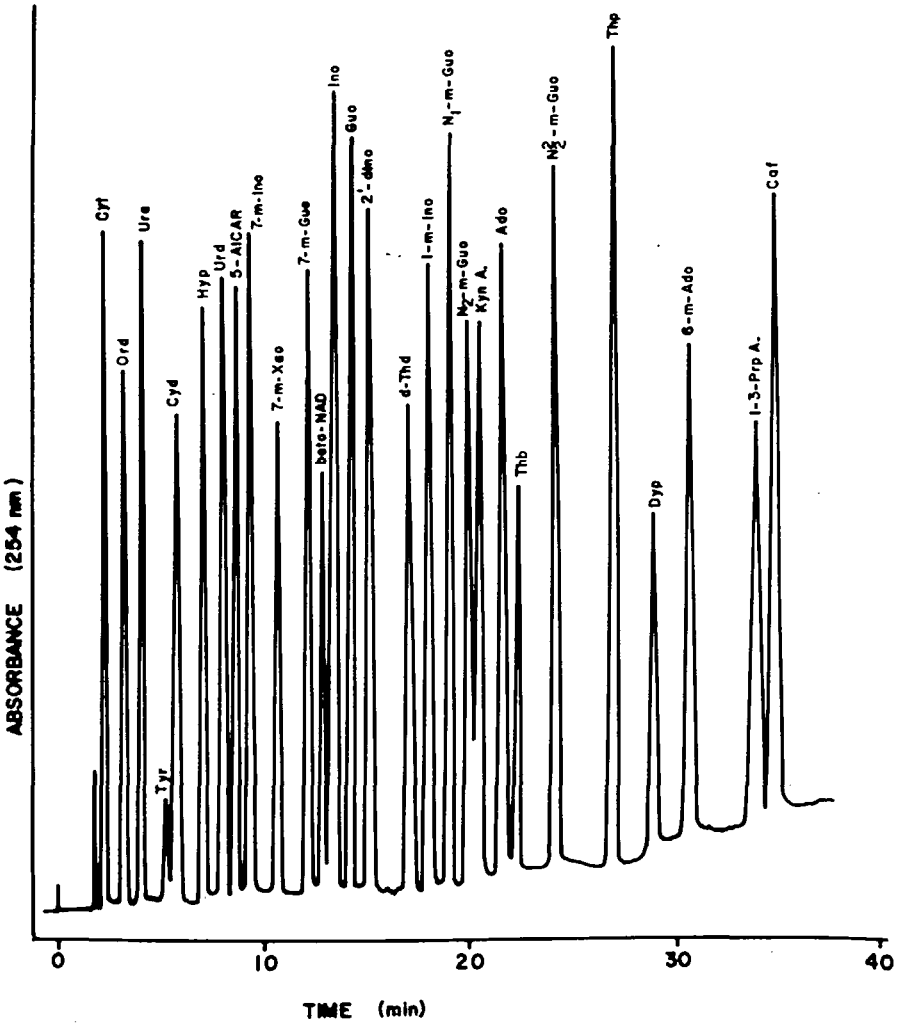

FIGURE 16. Gradient elution separation of 29 nucleosides, bases, and other compounds on a chemically bonded reversed-phase column. Column: Microbondapak® C_{18}; chromatographic conditions: low strength eluent: 0.01 KH_2O_4, pH 5.6; high strength eluent: 60% methanol/water (v/v); gradient: linear; flow rate: 1.5 m*l*/min; detection at 254 nm; sample size: approximately 0.5 nmol of each solute. (Reproduced from Hartwick, R. A., Assenga, S., and Brown, P. R., *J. Chromatogr.*, 186, 647 (1979). With permission.)

the solvophobic ion.[93] The use of the ion-pairing technique promised to reduce HPLC almost to a "one-column" type of chromatography, since a tremendous number of both ionized and nonionized species can be separated on the reversed-phase C_{18} columns now commonly used.

d. Gradient Optimization

An area of research which is still in its early stages, but which promises to be very fruitful for the chromatographer in coming years, is the area of the mathematical prediction of retention times. LC is a powerful separation tool, in part because of the complex interactions which occur within the column during a separation. The major thrust of the development of chromatographic theory for the past several decades has been in the area of column kinetics, i.e., in trying to identify and minimize those fac-

tors which lead to a broadening of the solute band as it traverses the column length. Because of the extreme complexity of the chemistry of the stationary phase-solute-mobile phase interactions, less success has been achieved in the elucidation of the liquid chromatographic process on a fundamental basis through which retention behavior might be predicted. Therefore, the control and the prediction of the retention times of compounds has remained a curious mixture of theory and art, where a successful separation is as much a result of educated approximations as it is of basic theory.

Ideally it may become possible in the future to predict the k′ values of selected compounds on an *a priori* basis. However, in the absence of the depth of understanding required to make such predictions, it is still possible to use empirical approaches which require only the observation of (rather than the interpretation of) the chromatographic behavior of solutes. The mathematical prediction of the retention times of solute bands under gradient conditions, based upon their isocratic behavior, can be a useful aid in the development of complex separations by gradient elution. Jandera and Churacek[94-96] and Schoenmakers et. al.[100] have been the most active in this field. Their work should be consulted for an in-depth review of the theory and mathematics of gradient optimization. A simplified outline of the salient features of the approach, as it applies to nucleosides and bases, is given below.

i. Derivation of Retention Equations

Assume that the k′ (where $k' = (T_r - T_0)/T_0$) is a reproducible and measurable function of the volume fraction C of the organic (stronger) component of the mobile phase in a reversed-phase HPLC system, where C may be expressed in volume %, mole fraction, etc.; thus, $k' = f(C)$. During a gradient elution separation, the mobile phase composition C is a known function of time, i.e., $C = f(t)$. If both of these functions are known, then theoretically it is possible to relate the two functions and to derive the necessary net function which will relate the elution time f(t) to the gradient curve f(C).

The first researchers to recognize this and to derive the necessary equations were Jandera and Churacek.[94-96] Since then, others have derived similar equations for a variety of gradient systems.[97-101] The work from these earlier researchers could not be appreciated until the development of chemically bonded packings which offered the stability and intercolumn reproducibility required to utilize such predictive capabilities.

The reversed-phase columns used for nucleoside and base separations exhibit a relationship between the log of the k′ and the mobile phase composition which approximates a linear function over the most commonly used range of organic concentrations.[83,84,101] Figure 14 shows a plot of the ln k′ vs. the volume % of organic solvent in the mobile phase for a 10-μm C_{18} column, at a constant pH of 5.6. In the experimental work done in our laboratory, it appears that excellent linearity for the ln k′ is observed up to about 15% methanol for most of the nucleosides and bases. Thus, the function of k′ with mobile phase composition over this range becomes

$$\ln k' = \ln k_0' + m \cdot C \qquad (6)$$

By definition, the function demonstrated in Equation 6 assumes an increasing concentration of the stronger solvent, whichever that may be in the particular system in use, so that the slope of the plot shown in Figure 14 will always be negative. Thus, it is convenient to define a constant $A = -m$, in which case,

$$\ln k' = \ln k_0' - A \cdot C \qquad (7)$$

If such a linearity is not observable over the range of organic concentrations used in

the gradient separation, then a polynomial expression must be found which will accurately describe the function. This is the method used by Schoenmakers et al.[99]

The gradient function must be known and may be concave, linear, or convex. In practice, most equations have been derived assuming a linear gradient. Asuming a linear gradient, the concentration C of the mobile phase at any given time is

$$C = C_o + b \cdot t \tag{8}$$

where b is the slope of the gradient curve in %/min and C_o is the initial composition of the mobile phase.

As a first step in relating Equations 7 and 8, one can state that the rate of movement of a solute band through a column is

$$\frac{dv}{dt} = \frac{V_o}{T_r} = \frac{V_o}{T_r(t,v)} \tag{9}$$

where V_o is the void volume of the column, dv/dt is the instantaneous velocity of the solute band at position V and time t in the column where V_0 is the column volume. The differential in Equation 9 may be solved if the retention time as a function of mobile phase composition C and the composition of the mobile phase at any point V in the column are known. Since both of these functions are known by Equations 7 and 8, Equation 9 is solvable. The critical assumption in any derivation such as this is that the instantaneous velocity at any point in the column is a function only of the solvent composition at that point, i.e., the solute band is always in equilibration with the changing mobile phase composition.

For the general case in which the sample is eluted before the completion of the gradient,

$$T_r = \frac{V_o + V_p}{R} + \frac{1}{A \cdot b} \ln \left[1 + \frac{A \cdot b}{R} (V_o k_o' e^{-A \cdot C_o} - V_p)\right] \tag{10}$$

which is valid under the condition

$$V_o k_o' e^{-A \cdot C_o} > Vp \tag{11}$$

For this equation, R is the flow rate in mℓ/min, b is the gradient slope in %/min change, and V_p is the delay volume created when the point of mixing of the gradient is removed from the point of injection. V_p usually arises from any mixing chambers or tubing that the eluent must pass through before reaching the injection port. A k'_o are two constants derived from the empirical plot of ln k'_o vs. C. A is the negative of the slope and k'_o is the intercept or the k′ at 0% organic phase. Being empirical constants, A and k'_o must be found for each compound individually on a given column type and for a given organic solvent.

If the gradient delay V_p is sufficiently large and the retention of a compound is sufficiently short, then it is possible that a solute will be eluted before the first change in solvent composition can overtake the eluting compounds. Under such conditions, the predicted retention time of a compound is given by

$$T_R = \frac{V_o}{R} \cdot [k_o' e^{-A \cdot C_o} + 1] \quad (12)$$

which applies when

$$V_o k_o' e^{-A \cdot C_o} < V_p \quad (13)$$

Finally, a third condition is possible in which the linear gradient is completed before a solute is eluted. In such a case

$$T_R = \frac{V_o}{R}(k_o' e^{-A \cdot C_f} + 1) + \frac{V_p}{R} + \frac{C_f - C_o}{b} + \frac{e^{A(C_o - C_f)} - 1}{A \cdot b} - \frac{V_p e^{A(C_o - C_f)}}{R} \quad (14)$$

for

$$V_o > \frac{V_p e^{A \cdot C_o}}{k_o'} + \frac{R(e^{A \cdot C_f} - e^{A \cdot C_o})}{A \cdot b \cdot k_o'} \quad (15)$$

where C_f is the final concentration of the mobile phase.

Thus, Equations 10, 12, and 14 may be used to predict the retention time of a compound with a linear ln k′ vs. C plot under linear gradient of any slope b. It is necessary to know only the A and k'_o values for the compounds on the column type being used. When used in conjunction with some relatively simple computer programs, it is possible to predict the retention times and thus the separation of a mixture of any complexity. The alternative to the use of this mathematical approach is to develop a gradient separation empirically by changing the gradient slope, flow rates, delay time, and final gradient conditions. This can be extremely time consuming and may not result in the discovery of the global minimum for the system.

ii. Application of Retention Equations

Figure 17 shows a computer-calculated plot of the predicted retention time of several nucleosides and bases as a function of gradient slope b. The other conditions of Equation 10 were kept constant. Figure 18 shows the actual chromatograms of these same compounds when chromatographed under these conditions. From both Figures 17 and 18, the usefulness of the derived equations is evident. The equations predict that no separation between uridine and xanthine will occur at a gradient slope of 0.5, but that at a slope of about 2.5, a separation will occur. In addition, the overall separation time will be reduced from 18 to 11 min. Without the aid of a plot such as Figure 17, one might not have suspected that increasing the gradient slope would increase the separation of two compounds not resolved, or to what degree the slope could be changed without causing other solute bands to overlap.

The main limitations to the full, unrestricted use of these equations are (1) column stability, (2) intercolumn variations, and (3) the prediction of peak widths under gradient conditions. Because the solute band resides in different compositions of the mobile phase throughout its distribution, the latter portion of the solute band is accelerated and the peak narrowed under gradient conditions. This effect was first noticed by Giddings.[102] Quantification of this compressive effect is not predictable with current

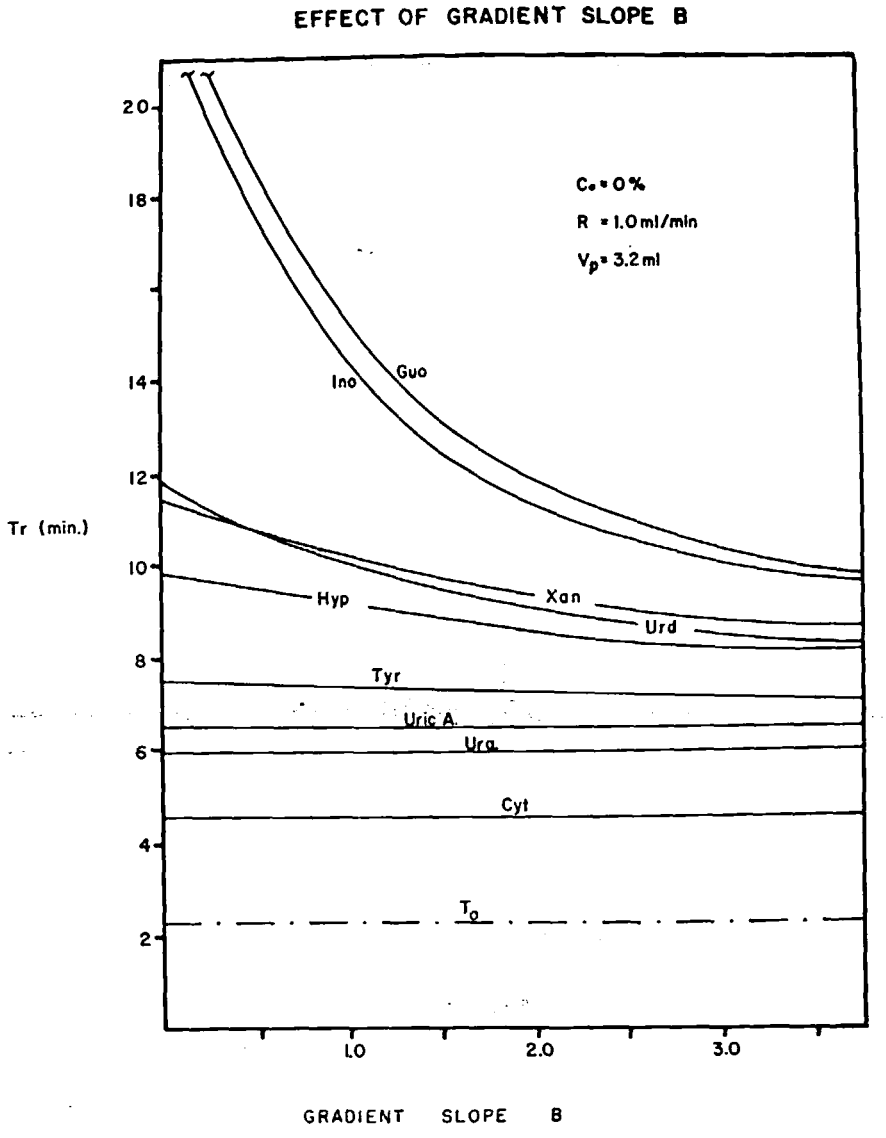

FIGURE 17. Plot of the calculated retention times of the nucleosides and other compounds against the gradient slope B. The gradient delay, flow rate, and initial composition were held constant. (Reprinted with permission from Hartwick, R. A., Grill, C. M., and Brown, P. R., *Anal. Chem.*, 51, 34 (1979). Copyright by the American Chemical Society.)

theory; therefore, one must simply assume an average solute band width for the compounds on a given type of column to determine when the resolution of adjacent bands becomes insufficient.

A major limitation to the empirical approach taken here is that of column variation. Although column technology has advanced tremendously, there are still large differences in selectivity between the columns of various manufacturers. Therefore, a set of retention data will only be valid for a given manufacturer's column type, unless experiment shows a close similarity of retention behavior for another packing material. As part of a comprehensive effort in this laboratory directed towards the analysis of nucleosides and other low molecular weight compounds, the retention data of 52 reference compounds was compiled for the column type given in the legend. Table 3 pre-

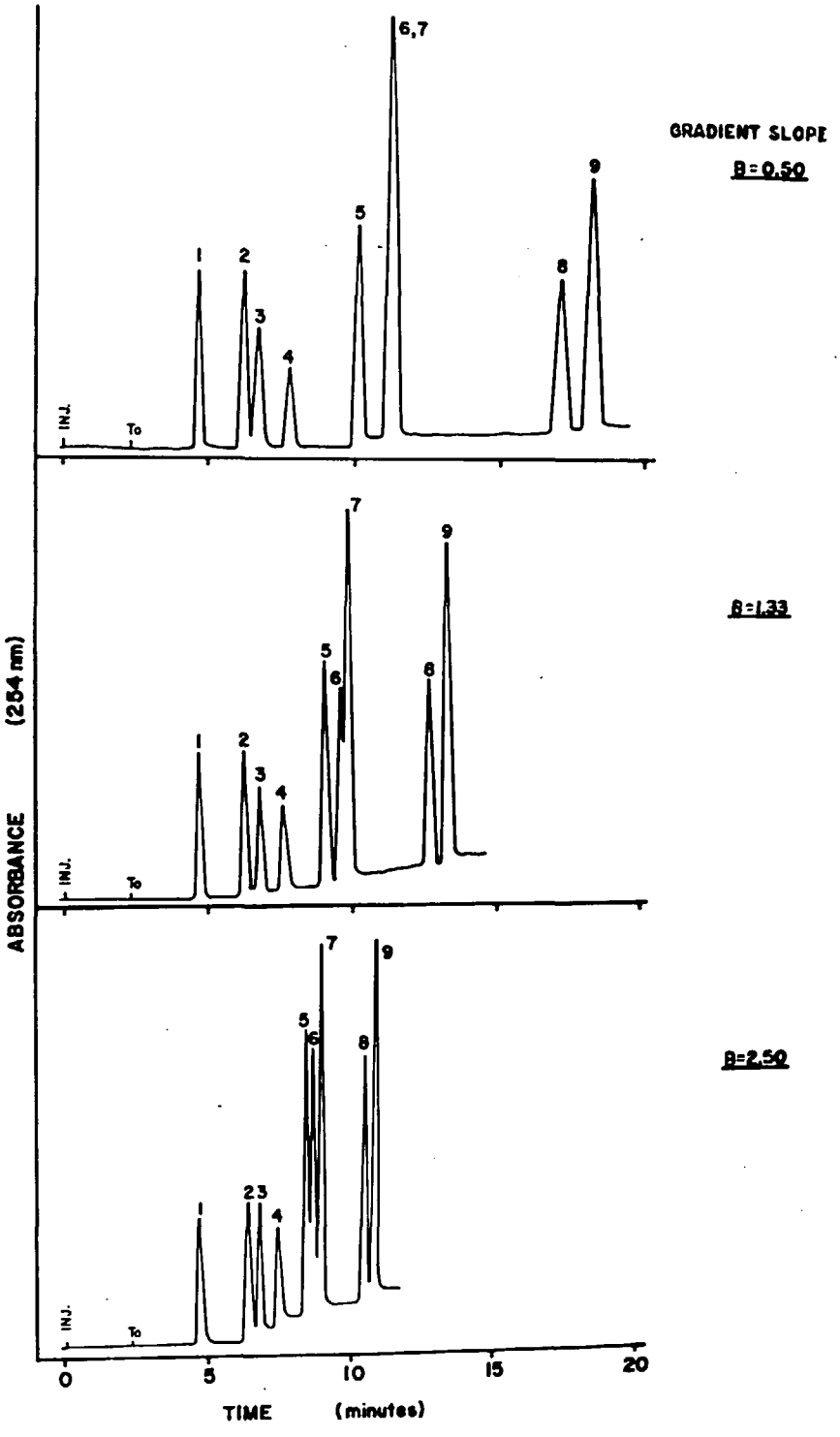

FIGURE 18. Chromatograms of the same compounds plotted in Figure 17 under three different gradient slopes. The slope of 2.50 %/min produced the best resolution of the compounds in the minimum time, as predicted by Figure 17. (Reproduced with permission from Hartwick, R. A., Grill, C. M., and Brown, P. R., *Anal. Chem.*, 51, 34 (1979). Copyright by the American Chemical Society.)

Table 3
SUMMARY OF RETENTION DATA AND SPECTRAL PROPERTIES FOR THE NUCLEOSIDES, BASES, AND OTHER BIOLOGICALLY IMPORTANT UV-ABSORBING COMPOUNDS[a]

Compounds	Observed retention time ± (Z)[b]	Predicted retention time ± (Z)[b]	Peak height ratios (1 S,D); 280 nm/254 nm	FL[c]	Retention behavior	
					A (1 SD)	k'_0 (1 SD)
Cytosine (CYT)	2.28 ± 0.04	2.08 ± 0.03	0.437 (0.0280)	—	0.0580 (0.001)	0.250 (0.002)
Orotidine (ORD)	3.25 ± 0.03	2.72 ± 0.02	0.516 (0.0240)	—	0.0330 (0.000)	0.63 (0.002)
Creatinine (CRT)	3.59 ± 0.06	3.92 ± 0.24	0.001 (***)	—	0.0565 (0.011)	1.35 (0.105)
Uracil (URA)	4.13 ± 0.04	4.52 ± 0.07	0.118 (0.0180)	—	0.0653 (0.003)	1.71 (0.034)
4-amino-5-imidazole carboxamide (4-A-5-IC)	4.31 ± 0.13	4.38 ± 0.17	0.621 (0.0140)	—	0.0646 (0.007)	1.63 (0.082)
L-Tyrosine (L-TYR)	5.40 ± 0.07	5.89 ± 0.18	2.47 (0.181)	WK	0.0844 (0.002)	2.55 (0.038)
1-Methyladenine (1-M-ADE)	5.77 ± 0.10	5.26 ± 0.39	0.194 (0.0640)	—	0.0973 (0.014)	2.16 (0.204)
Cytidine (CYD)	5.85 ± 0.10	5.96 ± 0.14	0.683 (0.0241)	—	0.0984 (0.004)	2.60 (0.076)
Hypoxanthine (HYP)	7.31 ± 0.12	6.93 ± 0.27	0.010 (***)	—	0.0786 (0.007)	3.23 (0.145)
Guanine (GUA)	7.56 ± 0.06	7.43 ± 0.15	0.532 (0.0140)	—	0.0926 (0.003)	3.59 (0.083)
Uridine (URD)	8.27 ± 0.19	7.67 ± 0.72	0.215 (0.0120)	—	0.1120 (0.017)	3.80 (0.435)
Xanthine (XAN)	9.53 ± 0.05	8.04 ± 0.16	0.459 (0.0010)	—	0.0975 (0.003)	4.04 (0.092)
5-Amino-imidazolo-carboxamide-riboside (5-AICAR)	8.77 ± 0.17	9.07 ± 0.90	0.529 (0.0370)	—	0.1300 (0.019)	4.98 (0.638)
7-Methylinosine (7-M-INO)	9.46 ± 0.19	9.30 ± 1.63	0.286 (0.0130)	ST	0.1730 (0.037)	5.40 (1.380)
DL-Kynurenine (DL-KYN)	9.82 ± 0.23	10.14 ± 0.73	0.036 (0.0014)	—	0.0938 (0.012)	5.69 (0.477)
L-Phenylalanine (L-PH-ALA)	10.40 ± 0.16	9.93 ± 0.71	0.001 (***)	WK	0.0759 (0.011)	5.40 (0.407)
Allopurinol (ALLO-PUR)	10.52 ± 0.21	10.30 ± 0.85	0.023 (0.0105)	—	0.1070 (0.015)	5.93 (0.600)
7-Methylxanthosine (7-M-XAO)	10.69 ± 0.21	10.34 ± 1.06	0.707 (0.0080)	WK	0.1590 (0.022)	6.41 (0.955)
3′,5′-Cylic CMP (C-CMP)	10.76 ± 0.25	10.48 ± 0.03	0.630 (0.0320)	—	0.1510 (0.001)	6.49 (0.022)
5-Methylcytidine (5-M-CYD)	10.79 ± 0.19	10.14 ± 0.77	1.130 (0.0280)	—	0.1310 (0.015)	5.97 (0.601)
Purine	11.20 ± 0.31	11.31 ± 0.16	0.148 (0.0070)	—	0.1010 (0.002)	6.79 (0.114)
7-Methylguanosine (7-M-GUO)	11.82 ± 0.16	11.85 ± 0.62	0.572 (0.0050)	MD	0.1890 (0.013)	8.74 (0.771)
Pyrimidine (PRMD)	11.92 ± 0.25	12.33 ± 1.49	0.319 (0.0250)	—	0.1050 (0.022)	7.85 (1.161)
Xanthosine (XAO)	12.40 ± 0.59	12.52 ± 1.08	0.512 (0.0215)	—	0.1670 (0.021)	9.27 (1.302)
3′,5′-Cyclic UMP (C-UMP)	12.86 ± 0.07	12.44 ± 1.16	0.171 (0.0040)	—	0.1510 (0.087)	8.82 (0.058)
β-Nicotinamide adenine dinucleotide (BETA-NAD)	13.00 ± 0.16	12.40 ± 1.91	0.120 (0.0060)	—	0.2120 (0.042)	10.14 (2.900)
Inosine (INO)	13.52 ± 0.16	13.53 ± 0.72	0.092 (0.0067)	—	0.1600 (0.013)	10.60 (0.930)
Adenine (ADE)	13.54 ± 0.15	13.61 ± 0.45	0.080 (0.0390)	—	0.1110 (0.006)	9.37 (0.416)
Guanosine (GUO)	14.40 ± 0.18	14.49 ± 0.68	0.373 (0.0020)	—	0.1620 (0.012)	12.26 (0.991)
7-Methylguanine (7-M-GUA)	14.65 ± 0.17	14.14 ± 0.89	1.120 (0.0460)	—	0.1290 (0.014)	10.55 (0.988)
3′,5′-Cyclic GMP (C-GMP)	15.12 ± 0.07	15.10 ± 0.52	0.397 (0.0412)	—	0.1690 (0.009)	13.69 (0.864)
N_2-Methylguanine (N_2-M-GUA)	15.22 ± 0.22	14.89 ± 1.13	0.535 (0.0170)	—	0.1330 (0.017)	11.76 (1.391)

Table 3 (continued)
SUMMARY OF RETENTION DATA AND SPECTRAL PROPERTIES FOR THE NUCLEOSIDES, BASES, AND OTHER BIOLOGICALLY IMPORTANT UV-ABSORBING COMPOUNDS[a]

Compounds	Observed retention time ± (Z)[b]	Predicted retention time ± (Z)[b]	Peak height ratios (1 S,D); 280 nm/254 nm	FL[c]	Retention behavior	
					A (1 SD)	k'_0 (1 SD)
3',5'-Cyclic IMP (C-IMP)	15.92 ± 0.06	15.59 ± 0.03	0.113 (0.0380)	—	0.1790 (0.001)	15.26 (0.053)
Hippuric acid (HIPP-A)	15.98 ± 0.05	15.70 ± 0.50	0.001 (***[d])	—	0.1090 (0.007)	11.93 (0.535)
Deoxy-thymidine (D-THYD)	17.29 ± 0.11	17.31 ± 1.09	0.565 (0.0260)	—	0.1550 (0.017)	17.46 (2.066)
L-Tryptophan (L-TRP)	17.47 ± 0.16	17.35 ± 0.11	1.430 (0.0480)	ST	0.1230 (0.001)	15.20 (0.150)
1-Methylinosine (1-M-INO)	18.22 ± 0.14	18.65 ± 0.40	0.171 (0.0880)	—	0.1930 (0.007)	25.49 (1.257)
6-Methylpurine (6-M-PUR)	18.98 ± 0.19	20.43 ± 1.32	0.063 (0.0020)	—	0.1240 (0.012)	21.37 (2.508)
2-Methyladenine (2-M-ADE)	19.13 ± 0.04	20.34 ± 0.39	0.129 (0.0050)	—	0.1260 (0.005)	21.42 (0.739)
N_1-Methylguanosine (N_1-M-GUO)	19.22 ± 0.18	19.63 ± 0.61	0.373 (0.0140)	—	0.1910 (0.011)	29.01 (2.145)
Purine riboside (PUR RIB)	19.69 ± 0.16	19.51 ± 0.49	0.099 (0.0208)	—	0.1690 (0.008)	25.01 (1.356)
Tubercydin (TUBR)	19.73 ± 0.15	19.50 ± 1.15	0.968 (0.0090)	—	0.1610 (0.018)	23.83 (2.943)
7-Methyladenine (7-M-ADE)	19.92 ± 0.17	20.09 ± 0.06	0.126 (0.0110)	—	0.1240 (0.001)	20.61 (0.102)
N_2-Methylguanosine (N_2-M-GUO)	20.23 ± 0.17	20.38 ± 1.44	0.441 (0.0450)	—	0.1910 (0.025)	32.30 (5.575)
Kynurenic acid (KYN A)	20.80 ± 0.16	21.46 ± 0.17	0.099 (0.0101)	WK	0.1290 (0.002)	24.59 (0.364)
Adenosine (ADO)	21.77 ± 0.22	22.55 ± 0.88	0.084 (0.0048)	—	0.1580 (0.009)	34.01 (3.030)
Theobromine (THB)	22.51 ± 0.13	23.52 ± 1.03	1.270 (0.0290)	—	0.1520 (0.011)	36.42(3.678)
3',5'-Cyclic-AMP (C-AMP)	23.47 ± 0.31	23.05 ± 0.26	0.125 (0.0140)	—	0.1880 (0.004)	45.80 (1.356)
N_2,N_2-Dimethylquanosine (N_2,N_2M-GUO)	24.42 ± 0.15	24.47 ± 0.31	0.476 (0.0070)	—	0.1550 (0.003)	41.64 (1.259)
6-Methyladenine (6-M-ADE)	24.90 ± 0.15	25.09 ± 1.39	0.465 (0.0060)	—	0.1450 (0.019)	40.99 (5.255)
6-Methyladenosine (6-M-ADO)	30.86 ± 0.27	28.38 ± 1.32	0.640 (0.0470)	—	0.2410 (0.017)	198.30 (36.450)
Caffeine (CAF)	35.08 ± 0.27	35.44 ± 1.87	1.260 (0.0561)	—	0.1420 (0.017)	116.80 (17.520)

[a] Log data valid over the range of 0 to 15% methanol, pH 5.6. Column type: μBondapak C_{18} (Waters Associates), 10 μm particle diameter.
[b] Error limits calculated as $\pm s_i - t_{\alpha,f} / \sqrt{n}$ at $\alpha = 0.10$.
[c] Fluorescence symbols: WK, weakly fluorescent; ST, strongly fluorescent.
[d] Numbers too small to be accurately determined.

sents the retention times, absorbance ratios, and fluorescence response of these compounds, in addition to the log plot data, which will allow for the calculation of predicted retention times under any elution conditions. The correlation between the predicted and the observed retention times in Table 3 is shown in Figure 19. The slope of 0.996 and correlation coefficient of 0.998 shows the excellent results obtained by the predictive equations. While mathematical gradient optimization in its present form is not exact, within a given research problem it can be quite useful for finding (at least

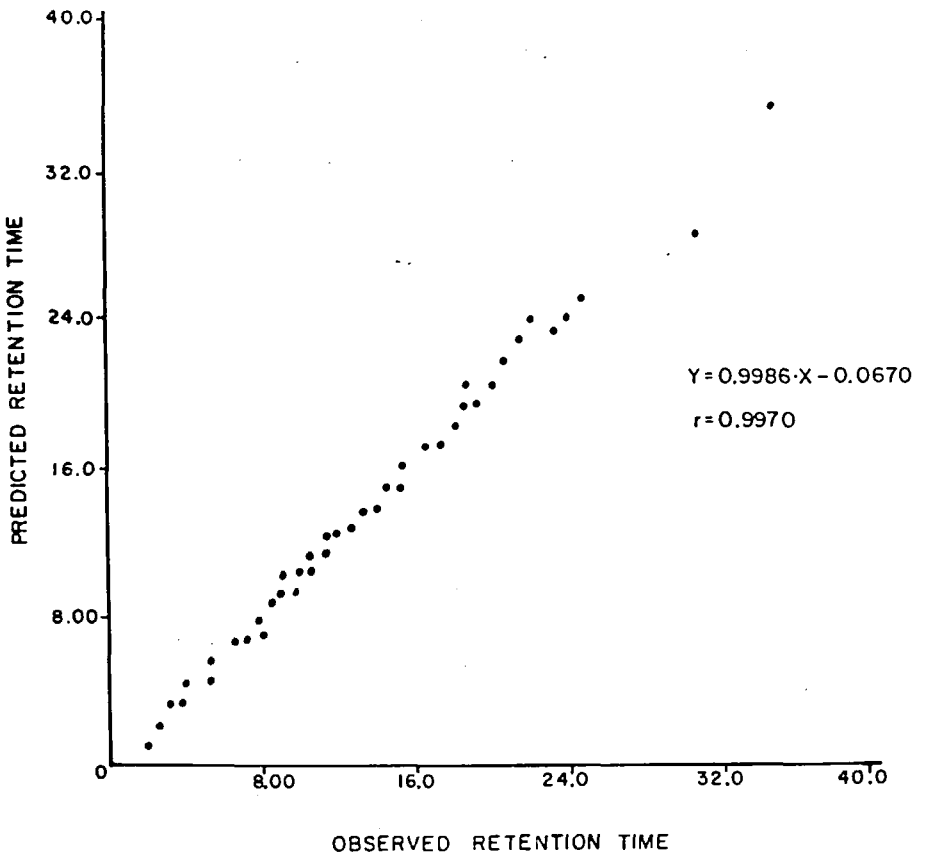

FIGURE 19. Correlation between the observed and the predicted retention times for 42 of the nucleosides, bases, aromatic amino acids, and other compounds on a chemically bonded reversed-phase column. The retention times were calculated from the equations described in the text. (Reproduced from Hartwick, R. A., Assenga, S., and Brown, P. R., *J. Chromatogr.*, 186, 647, (1979). With permission.)

approximately) the best gradient conditions for a given separation within the limits discussed.

e. Reproducibility of Retention Times

The chemical stability of bonded phase columns is generally excellent, which allows for excellent intra- and intercolumn reproducibility. The reproducibility of peak areas is a function of the detection and integration system more than of the chromatographic column itself.

Table 4 shows the run-to-run and the day-to-day precision observed for the retention times of five of the nucleosides and bases on a reversed-phase column using gradient elution conditions. The relative SD of the replicate injections averaged about 1.5%, while the day to day variations over a 2-week period averaged slightly higher, at about 2.2% relative SD (RSD). These variations will be somewhat less when isocratic rather than gradient elution is used.

Table 4 also shows the column-to-column reproducibility observed for the retention times of these same compounds on four different columns, all of which had been used for varying lengths of time. The RSDs for these data averaged just under 7%. A realistic estimate for the overall variability between columns appears to be in the range of 10 to 15%.

Table 4A
RUN-TO-RUN PRECISION (SINGLE COLUMN) WITHIN 1 DAY

Compound	Retention time (min)	SD (n = 5)	RSD (%)	Range (+/− 2 SD)
Hyp	7.47	0.112	1.50	7.25—7.69
Ino	14.2	0.190	1.34	13.8—14.6
Guo	15.1	0.201	1.33	14.7-15.5
Trp	17.6	0.263	1.50	17.1-18.1
Caf	35.7	0.527	1.47	34.6-37.8
	Day-to-Day Precision (Single Column) Over 14 Days			
Hyp	7.40	0.252	3.46	6.90—7.90
Ino	14.1	0.315	2.23	13.5—14.7
Guo	15.0	0.319	2.13	14.4—15.6
Trp	17.4	0.322	1.85	16.8—18.0
Caf	35.5	0.417	1.17	34.7—36.3
	Column-to-Column Reproducibility			
Hyp	7.20	0.550	7.63	6.1—8.3
Ino	13.7	0.992	7.24	11.7—15.7
Guo	14.6	1.03	7.05	12.5—16.7
Trp	16.9	1.10	6.51	14.7—19.1
Caf	34.8	1.28	3.69	32.3—37.4

Table 4B
PRECISION OF QUANTITATION FOR HUMAN SERUM

Compound	Average sample conc (μmol/l)	SD*	RSD (%)
Creatinine	76.4	(2.38)	3.11
Uric acid	22.1	(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

* SD for five replicate injections are given in parentheses.

f. Quantitation and Lower Detection Limits

When using electronic integrators and well-designed detectors, excellent reproducibility of peak areas is possible. Table 4 shows the precision observed by Gehrke et al.[77] for the HPLC analysis of six of the nucleosides using the internal standard method. An average coefficient of variation of 1.5% was found.

The lower limits of detection will be a function of several factors: (1) system noise, (2) detection wavelength (compound response factor), (3) resolution from adjacent peaks, and (4) column efficiency. At a wavelength of 254 nm, the detection limits for UV-absorbing nucleosides and bases (molar absorptivity 10,000 to 20,000) are generally in the range of 10 to 50 pmol. This limit can be lowered if fluorescence detection is utilized with derivatization. Kuttesch et al.[103] were able to detect as little as 2 pmol of the 1,N_6-etheno derivative of adenosine in cellular extracts using fluorescent detection.

Table 5
PARTIAL SUMMARY OF DEPROTEINIZATION METHODS FOR BIOLOGICAL SAMPLES

Principle	Example
Dielectric change	Acetonitrile or ethanol
Temperature change	Boiling water bath
Specific complexation or adsorption	Borate gels, precolumn conc
pH change	TCA and PCA
Ionic strength	Ammonium sulfate
Filtration	Ultrafiltration with membranes

III. SAMPLE PREPARATION

A. Review of the Methods

The tremendous increases in the sensitivity of HPLC analyses of nucleosides and bases have necessitated a re-evaluation of the commonly used sample preparation techniques. Many of the commonly used protein precipitation techniques are incompatible with the picomole levels of analyses made possible by HPLC. Therefore sample preparation techniques which are more compatible with the demands of HPLC have recently been evaluated (Table 5).

For most HPLC analyses of biological fluids, the primary requirement of the sample preparation technique will be the removal of proteinaceous material from the sample. Such materials must be removed prior to sample injection in order to prevent irreversible adsorption to the packing and column plugging by the proteins.

Perhaps the most widely known of the protein precipitation techniques is the trichloroacetic acid (TCA) and perchloric acid (PCA) precipitation methods. The combination of high ionic strength and low pH produced by these acids makes them highly effective precipitation agents. The excess acids or salts in the sample must be removed, either by extraction (TCA) or by precipitation as insoluble potassium salts (PCA). The traditional method of extracting excess TCA has been by the use of ether extractions. Recently, Khym[104] introduced a novel method using a water-insoluble Freon®-amine solution to extract the remaining TCA. This technique was found to be compatible with nucleotide analysis on microparticulate packings,[105,106] but was found to be less suited for nucleosides and bases.[107]

Another method available for the deproteinization of biological fluids is the addition of an organic solvent to the sample. A decrease in the dielectric constant of the solution results in the aglomeration of many of the larger serum proteins. Acetonitrile, methanol, and ethanol are frequently used solvents. Many rapid and simple assays for drugs in serum have been developed using the organic precipitation technique. However, for the analysis of serum nucleosides and bases, organic precipitation appears to be less useful since many of the nucleosides and bases are insoluble in organic solvents.

An increase in temperature will cause rupture of the secondary structure of serum proteins, resulting in their precipitation from solution. However, the application of heat to serum often results in a gel formation of the proteins, leaving the serum in a form unsuitable for quantitative analysis. It can be a useful sample preparation technique for the analysis of the formed elements,[108] although TCA and PCA are often more versatile. Another limitation of heat denaturation is that many biological compounds are thermally labile, so that spurious high or low values may be encountered unless proper precautions are taken by analyzing parallel blanks.

A very mild method of protein removal is by the addition of ammonium sulfate or other innocuous salts to the sample. This "salting out" effect is usually reversible, and is often used where the proteins or enzymes must be recovered and reconstituted.

Proteins of large molecular weights may be filtered from the sample using ultrafiltration membranes. A minimum molecular weight of 10,000 to 25,000 is usually filtered by the membrane using either pneumatic or centrifugal force to push the sample through the membrane at reasonable rates. Ultrafiltration is extremely simple and clean since no extraneous chemicals are added to the sample. In addition, the sample is not diluted. In ultrafiltration, only free compounds will be analyzed. Thus, for solutes bound to serum proteins, such as tryptophan,[109] only a fraction of the total amount present will be measured.

A simple yet versatile method of sample preparation is that of a sample precolumn used outside of the instrument. Uziel developed a borate-affinity column[110] that proved to be useful for the selective analysis of ribonucleosides and other cis-diol compounds.[77,79] In this method, the sample is passed through the column, with the compounds of interest being retained on the column. The retained compounds are then eluted with a suitable eluent. The precolumn need not contain a selective affinant. Several companies have recently introduced small cartridges packed with reversed-phase material which allow for a wide range of compounds to be retained by varying the eluents used.

There are several factors which must be considered in choosing any sample preparation technique. The analysis of nucleic acid components in biological fluids requires a high sensitivity, often in the picomole range. Therefore, any sample preparation technique chosen should be chemically clean and minimize the introduction of extraneous material which might interfere with the analysis. If possible, the method should be simple, with a minimum of steps in which the sample is subject to dilutions or loss of material. In addition, any method chosen should be rapid enough for a reasonable sample throughput and be inexpensive enough for routine work. Depending upon the problem at hand, some of these requirements may be less important than others; however, the goal of any technique is to eliminate interfering compounds while leaving unaltered the components of analytical interest.

B. Evaluation of Methods

1. Borate Columns

The $B(OH)_3$ molecule has long been known to complex specifically with cis-diol.[111] Uziel developed a polyacrylamide-borate gel which proved to be very useful in a precolumn for the selective retention of the ribonucleosides.[110] Figure 20 shows the structure of this gel and the mechanism of the retention. Complexation occurs at alkaline pH values of pH 8 or greater.

Gehrke and co-workers[77,79] utilized this gel as a basis for the selective analysis of the ribonucleosides in biological fluids, especially in urine. The sample is made alkaline by adding 0.1 m*l* of 2.5 mol/*l* ammonium acetate (pH 8.8) to 1.0 m*l* of urine. After repeated washings, the column is eluted with 5 m*l* of 0.1 mol/*l* formic acid. An internal standard (N_2,N_2-dimethylguanosine) is then added to the eluate, which is subsequently shell frozen, lyophilized, and reconstituted in 1.0 m*l* of water or buffer.

Figure 21 shows a reversed-phase HPLC separation of a sample of pooled urine to which had been added standard nucleosides. The sample had been prepared using the borate technique. The lack of interferences in the normally complex urine matrix is evidence of the high specificity of this preparation technique.

The recoveries of nucleosides added to urine was 87 to 100%, as shown by the data presented in Table 6. The relative SD was approximately 4%, illustrating the excellent reproducibility of the method as applied to urine samples.

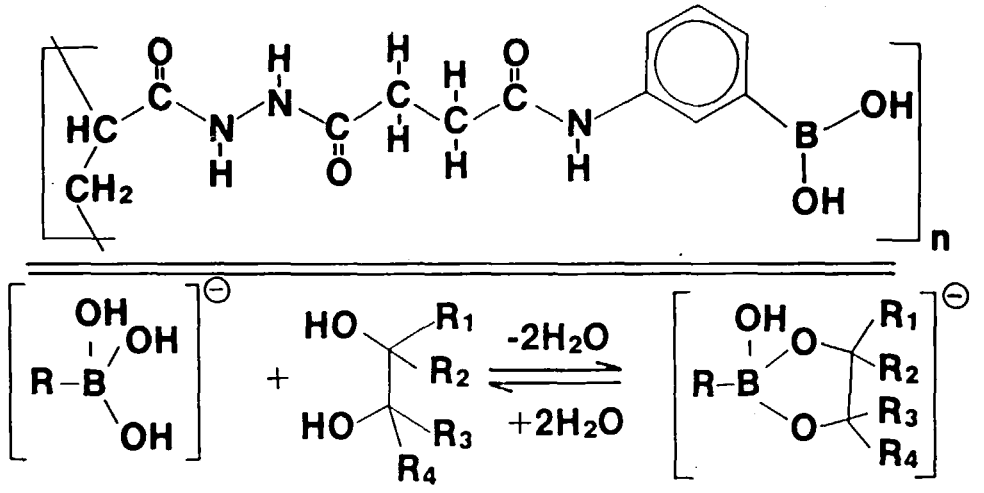

FIGURE 20. Top: the structure of boronate derivatized polymer. Bottom: formation of cis-diol boronate complex. Complex formation is reversibly favored under alkaline conditions and is highly specific for cis-diol groups. (Reproduced from Davis, G. E., Suits, R. D., Kuo, K. L., Gehrke, L. W., Waalkes, T. P., and Borek, E., *Clin. Chem. (Winston-Salem, N.C.)*, 23(8), 1427 (1977). With permission.)

The use of such group-specific sample preparation procedures has the advantage of greatly reducing the demands made upon the chromatographic technique. In addition, the precolumn can function as a sample concentration method, thus increasing the sensitivity of the analysis. However, there are times when it is necessary to analyze a sample for compounds belonging to different chemical groups. In such cases, the use of highly specific affinants can be a disadvantage. Sample cartridges packed with polar and nonpolar stationary phases have recently become available from several manufacturers and offer an alternative to specific affinants.

2. Comparison of Techniques

A comparison of the TCA, ammonium sulfate, ultrafiltration, and sample cartridge (precolumn isolation) techniques has recently been made[107] for use prior to the HPLC analysis of nucleosides, bases, and other small molecular weight, UV-absorbing compounds commonly found in human serum. These methods were investigated with regard to the efficiency of recovery, reproducibility, and general suitability for the determination of the profiles of nucleosides, bases, and other endogenous UV-absorbing compounds in serum.

a. TCA

The TCA method for sample preparation is widely used to precipitate the proteins in various biological samples. However, it proved to be less suitable for the analysis of nucleosides and bases at the nanomole-picomole level in human serum. Table 7 shows a summary of the recoveries observed for nucleosides, bases, and the aromatic amino acid tryptophan using several different sample preparation techniques.

In general, the recoveries of added nucleosides in serum was poor using either 6 or 12% TCA as the denaturant. Recoveries ranged between 54 to 68%. It was found that about 15% of the loss could be attributed to the partial solubility of the nucleosides in the amine-Freon® solution itself, which is used to extract the TCA after precipitation.

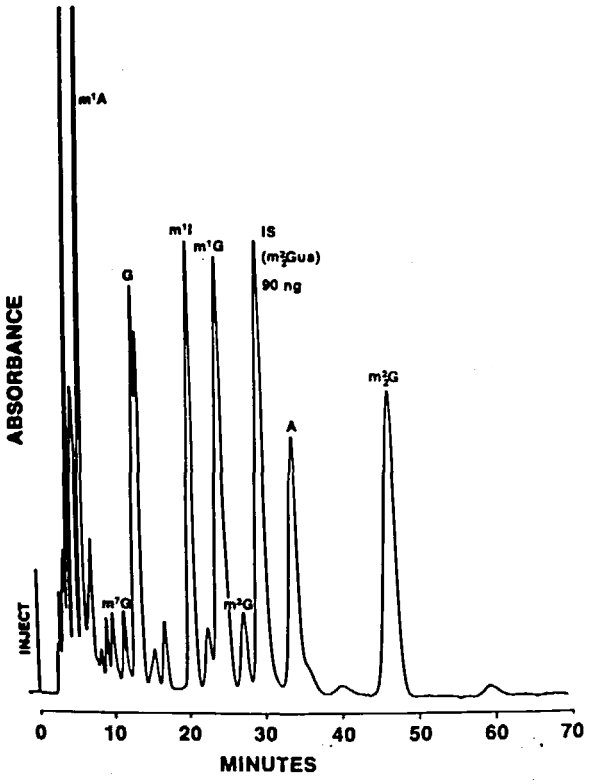

FIGURE 21. Reversed-phase, isocratic separation of nucleosides added to pooled urine after boronate-gel extraction. Samples: 50 μl of extract, equivalent to 25 μl of urine, with 125 pmol of each of the six nucleosides added. Chromatographic conditions: same as in Figure 13. (Reproduced from Davis, G. E., Suits, R. D., Kuo, K. L., Gehrke, L. W., Waalkes, T. P., and Borek, E., *Clin. Chem. (Winston-Salem, N.C.)*, 23(8), 1427 (1977). With permission.)

Table 6A
ANALYTICAL RECOVERY OF NUCLEOSIDES ADDED TO POOLED CONTROL URINE

Nucleoside	Urine + supplement	Urine	Supplement found	Average recovery (%)
	nmol/ml[a]			
M¹A	23.30	17.38	5.92	92
m²G	10.05	5.69	4.36	88
G[b]	13.47	8.91	4.56	92
m¹I	15.46	10.48	4.98	99
m¹G	10.66	5.64	5.02	101
m³G	10.69	5.46	5.23	100
A	7.38	2.55	4.83	98
m_2^2G	15.72	11.28	4.44	87

[a] Each value is an average of three runs.
[b] An unknown peak eluted with G. Both were integrated together.
[c] Identity of this peak is based on retention time only.

Table 6B
PRECISION OF HPLC ANALYSIS FOR URINARY NUCLEOSIDES[a]

Nucleoside	Mean	SD	CV (%)
	nmol/ml		
ψ	225.3	4.07	1.8
m¹A	15.18	0.54	3.5
m⁷G[b]	6.72	0.31	4.7
m¹I	10.26	0.35	3.4
m¹G	5.69	0.16	2.8
m²G	5.52	0.23	4.1
A	2.52	0.15	6.0
m_2^2G	11.37	0.82	7.2

[a] Each value is the average of four independent runs with four different affinity columns and a pooled urine control.
[b] Identity based on retention time only; needs further confirmation by other methods.

Table 7
RECOVERIES OF COMPOUNDS ADDED TO POOLED HUMAN SERUM[a]

Compound	TCA at 6%	TCA at 12%	$(NH_4)_2SO_4$	Ultrafiltration at pH 7.8	Ultrafiltration at pH 5.1	Reversed-phase cartridge
Xanthosine[b]	58.8 ± 6.2	66.4 ± 1.0	97.4 ± 7.7	99.3 ± 2.5	74.8 ± 4.2	—[c]
Inosine	59.4 ± 7.7	65.9 ± 2.7	95.6 ± 7.6	98.9 ± 2.2	97.7 ± 1.7	101 ± 2.85
Guanosine	54.0 ± 4.7	67.6 ± 0.3	84.8 ± 9.6	73.6 ± 3.9	85.9 ± 6.4	92.0 ± 5.32
Tryptophan	—	—	102 ± 12.6	12.1 ± 2.6	96.2 ± 3.7	43.3 ± 2.15
Theophylline	—	—	82.7 ± 5.1	40.7 ± 5.0	85.5 ± 4.7	91.2 ± 4.8
Theobromine	—	—	73.1 ± 7.5	81.3 ± 3.2	83.2 ± 4.3	90.2 ± 5.4
Caffeine	—	—	88.9 ± 6.7	83.0 ± 9.1	87.7 ± 4.5	92.8 ± 5.7

[a] Recoveries are listed as % and are an average of five replicate samples.
[b] All ranges are reported at the 95% confidence level.
[c] Variable recovery, pH dependent.

b. Ammonium Sulfate

The ammonium sulfate technique proved to be more suitable for the removal of serum proteins. In practice, an equal volume of serum and saturated ammonium sulfate solution are mixed and centrifuged. The supernatant is filtered through a membrane filter (0.22 μm). Table 7 shows that recoveries ranging from 73 to 100% were observed for nucleosides and bases added to the control serum matrix.

c. Ultrafiltration

Ultrafiltration of sera may be accomplished rapidly and cleanly using centrifuge cones and a bench-top laboratory centrifuge. For the study presented in Table 7, cones

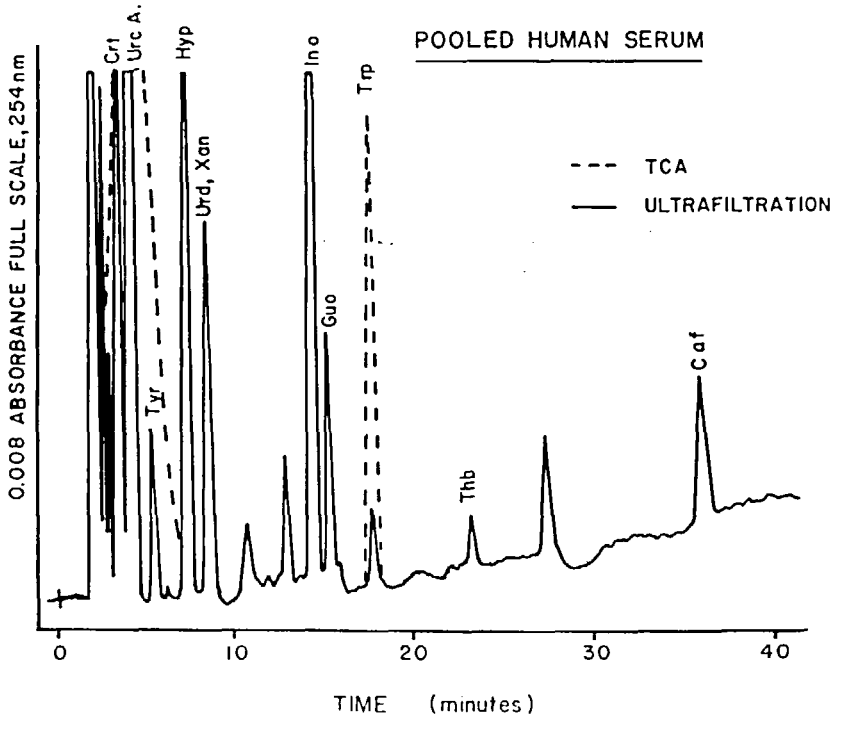

FIGURE 22. Chromatograms showing the typical nucleoside and base profile observed in normal sera prepared using the TCA and the ultrafiltration sample preparation techniques. The ultrafiltration method produced the cleanest chromatograms of several methods evaluated, although only the free fraction of components bound to serum proteins will be measured. Sample: pooled human serum, with no standards added. Chromatographic conditions: same as those in Figure 16. (Reproduced from Hartwick, R. A., Van Hauerbeke, D., and Brown, P. R., *J. Liq. Chromatogr.*, 2, 725 (1979). With permission.)

with a nominal molecular weight selectivity of 25,000 were used. A small volume of serum (about 1 m*l*) is added to the cone and centrifuged at moderate speed for 15 to 20 min. The ultrafiltrate is collected and analyzed without further preparation. Using the centrifuge, any number of samples may be prepared simultaneously so that the sample throughput can be quite high.

Ultrafiltration of control serum was performed at pH 5.1 and 7.8. At pH 7.8 excellent recoveries were observed for all compounds except tryptophan and theophylline. These two compounds showed recoveries of only 12.1 and 40.7%, respectively.

When the pH of the serum was adjusted to 5.1 before ultrafiltration, the recoveries of tryptophan and theophylline increased to 96.2 and 85.5%, respectively. In order to determine if this pH-dependent loss was due to adsorption to serum proteins or to the filter cones themselves, standard solutions at these pH values were ultrafiltered. Recoveries of almost 100% were observed for all compounds except theophylline, of which only 80% was recovered. The loss of tryptophan is consistent with the results of other researchers[109] who have found that tryptophan is reversibly bound to serum albumin, probably through the carboxylic acid group.[112]

Recoveries alone are not the only factor to be considered in selecting a method of sample preparation. Freedom from interferences and ease of use can also be critical factors. Figure 22 shows a chromatogram of the pooled serum processed by TCA superimposed upon the chromatogram of the same serum processed using ultrafiltration

cones. When using the TCA method, interferences are encountered during the first 5 to 7 min of the HPLC analysis. Because of these interferences, the concentrations of creatinine, uric acid, and tyrosine, all of which are endogenous to normal serum, cannot be determined in these samples.

d. Reversed-Phase Cartridges

In a manner similar to that described for the borate-gel columns, sera may be prepared by passing the samples through small columns packed with any of a variety of packing materials. Since the reversed-phase mode of HPLC has proven to be highly suitable for the separation of nucleosides and bases, the use of reversed-phase sample columns (cartridges) was investigated. Table 7 shows that excellent recoveries were obtained using the precolumn method. The samples were prepared by passing 1 m*l* of serum through the cartridge followed by 1 m*l* of 0.02*M*KH_2PO_4, pH 5.6. This is a weak eluent for the reversed-phase system and functions to flush the proteins from the column. The retained nucleosides and other compounds are eluted by passing 1 m*l* of 60% methanol through the column.

It was shown previously (in the chromatography section of this paper) that the retention times of nucleosides and bases on a reversed-phase column can be predicted for any set of conditions if the slope and the intercept of the ln k′ vs. composition of the mobile phase plots are known. In an analogous fashion, the elution conditions for the sample cartridges can be calculated so that the proper conditions can be determined which will selectively retain the compounds of interest on the sample cartridge. Equation 16

$$V_R = V_0(k'_0 e^{-AC} + 1) \qquad (16)$$

may be easily derived and allows one to calculate the predicted retention volume of the solute of interest under any conditions of mobile phase composition C. The V_0 of the cartridge is obtained by weighing, while the k'_0 and A values may be obtained using the reversed-phase packing material in a regular HPLC column.

Figure 23 shows the predicted methanol elution curves for four of the nucleosides and bases and for the compound creatinine. Referring to Figure 23, one can see that over 50 m*l* of aqueous buffer could be passed through the sample cartridge without the loss of any caffeine, while only 1.5 m*l* of the same buffer would be required to elute creatinine. All of the compounds studied would be rapidly eluted using a 60% methanol solution. Curves such as that shown in Figure 23 can be useful for selectively isolating groups of compounds provided that the differences in retention are sufficiently different.

3. Efficiency of Protein Removal

The relative efficiencies of protein removal were investigated[107] for the TCA, ammonium sulfate, ultrafiltration, and sample cartridge methods. The data presented in Table 8 show that all of the methods except that of ammonium sulfate removed essentially all of the serum protein (as measured by TCA precipitation using the Biuret technique). The addition of ammonium sulfate to serum resulted in the precipitation of only 65% of the protein. However, in our own laboratory, there were no detectable differences in the lifetimes of columns in which samples treated by ammonium sulfate were analyzed as opposed to the other methods.

C. Conclusions

The sample preparation techniques discussed are not exhaustive, but should be suf-

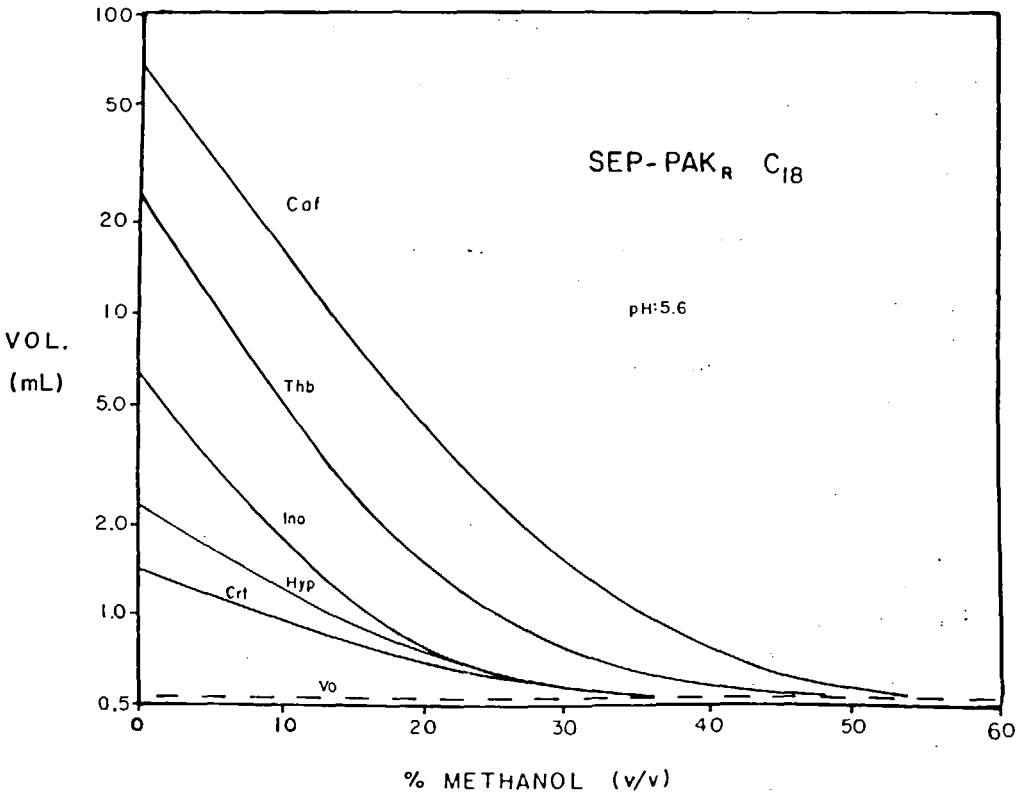

FIGURE 23. The minimum volumes predicted to be necessary to elute the compounds of interest from a small precolumn (Sep-Pak®) packed with reversed-phase material. The sample is forced through the cartridge using a syringe after which the appropriate volumes of a weak eluent (0% methanol) may be used to elute the nonretained proteins. This is followed by the appropriate volume of eluent of the correct methanol concentration to elute the desired compounds. (Reproduced from Hartwick, R. A., Van Hauerbecke, D., and Brown, P. R., *J. Liq. Chromatogr.*, 2, 725 (1979). With permission.)

Table 8
EFFICIENCY OF DEPROTEINIZATION METHODS

Method	Efficiency[a] (%)
TCA	100
Ammonium Sulfate	(65.5 ± 2.1)[b]
Ultrafiltration	100
Precolumn concentration	100[c]

[a] Biuret method of protein assay.
[b] 95% confidence level.
[c] Dependent upon elution conditions.

ficiently complete to allow the researcher to be aware of the limitations and advantages of each approach. The borate-gel method of Uziel and Gehrke has proven to be extremely compatible with the analysis of nucleosides in complex sample matrices. If it is desired to separate other compounds as well, then alternative methods must be used. Of the ones discussed, ultrafiltration has proven to be the most reliable and easy to

use although the methods or combinations of methods may be found to meet the specific requirements of the project at hand. It should be added that if small injection volumes are used, with a pre-column, then direct injection of urine or serum with no pretreatment is possible. While "ghosting" from sample protein bleed can occur, the simplicity of this approach is worth its use in certain cases.

IV. PEAK IDENTIFICATION

The improvements in column efficiency and detector sensitivity have made possible the routine separation of picomole quantities of the nucleosides and bases in complex biological matrices. However, this same sensitivity and efficiency has put new demands upon the methods used to identify the separated solute bands. Unless an extremely selective sample extraction technique is used, it is not possible to identify a peak in a complex biochemical mixture unambiguously on the basis of its retention time alone.[113]

In attempting to identify minute amounts of a separated compound, several approaches may be taken. One approach is to use a single, powerful technique such as mass spectrometry (MS), which has the potential to give absolute identification. However, the coupling of HPLC with MS has not been the panacea that the gas chromatography interface has been, although there has been some success in this area. The problem of applying MS to the identification of biological solutes goes beyond the problem of stripping the mobile phase from the sample. A major advantage of HPLC over gas-liquid chromatography (GLC) in biochemical work is that it is ideally suited to the separation of thermally-labile, nonvolatile compounds. These same compounds usually cannot be analyzed by MS without derivatization. Thus LC/MS, while very powerful in some applications, cannot always be easily used in peak identification of biological samples.

An alternative approach may be taken by using a series of less definitive identification techniques in order to gain insight into the presence of certain functional groups or structures. In working with biological samples, it is possible to take advantage of enzymes as identification tools as well as to use various spectral information and chemical and chromatographic behavior to determine with a high degree of confidence the identities of picomole quantities of separated compounds.

Another factor which will dictate which identification procedures are suitable in a particular project is the number of samples and peaks which must be identified. Peak collection, purification, and mass spectral analysis would be justified for several peaks which occurred in many samples or for important peaks in only one or two samples (provided there is sufficient material for the microgram needed). However, in studies where hundreds of samples must be analyzed, it is difficult to identify unambiguously every peak of every sample. In such studies, it is beneficial to have on-line techniques which can confirm the purity and identity of recurring peaks, as well as to provide information on the identities of new peaks that occur.

Table 9 summarizes some of the possible methods which can be utilized for peak identification. These will be discussed in detail, and applications presented for the identification of nucleosides and other low molecular weight compounds in bological samples.

A. Retention Data

The first step in any identification procedure is a match-up of retention times of the separated peak and a standard reference compound. This requires a knowledge of the biochemical system being studied as well as a certain degree of luck. Figure 24 shows a sample of human serum ultrafiltrate, separated on a reversed-phase column under

Table 9
SOME POSSIBLE METHODS OF PEAK IDENTIFICATION

Retention Data
Comparison of retention times with standards
Cochromatography with standards
Comparison of unknown and standard retention data under a wide variety of conditions (pH, eluent strength, gradient, column type)
On-Line Identification Aids
Absorbance ratios
UV-vis and fluorescence scans
Enzymatic peak shifts*
Chemical derivatization or complexation*
On-line LC/MS interface
Off-Line Techniques
Fraction collection, analysis by auxilliary methods (MS, IR, NMR, ORD, etc.)

* Requires at least two chromatographic runs.

gradient elution conditions. There is approximately 100 nmol of material contained in each peak. The first step in the identification of each peak was to compare the retention times of the serum compounds with those of standards run on the same type of column under the same chromatographic conditions. Usually the retention times will be reliable to 3 to 5% intracolumn and about 10 to 20% on an intercolumn basis, the retention time of the unknown may be similar to several different standards.

The use of relative retention data can sometimes be helpful in such fluctuations. Anderson and Murphy[73] have defined a relative retention parameter (RRP) as

$$\mathrm{RRP} = \frac{\mathrm{TR_{STD}} - \mathrm{TR_X}}{\mathrm{TR_{STD}}}$$

where TR_{STD} is the retention time of a standard and TR_X is the retention time of the compound of interest. They reported that this parameter was relatively insensitive to daily fluctuations in column behavior. These parameters were not constant, however, for varying concentrations of organic modifiers (reversed-phase system) or for greater than 20% variations in flow rates.

In order to determine whether or not there is an exact match-up between the standard and the unknown peak, the standard may be co-injected with the sample. Figure 25 shows the same serum sample of Figure 24 co-injected with the indicated compounds. Enough standard should be co-injected to increase markedly the peak of in-

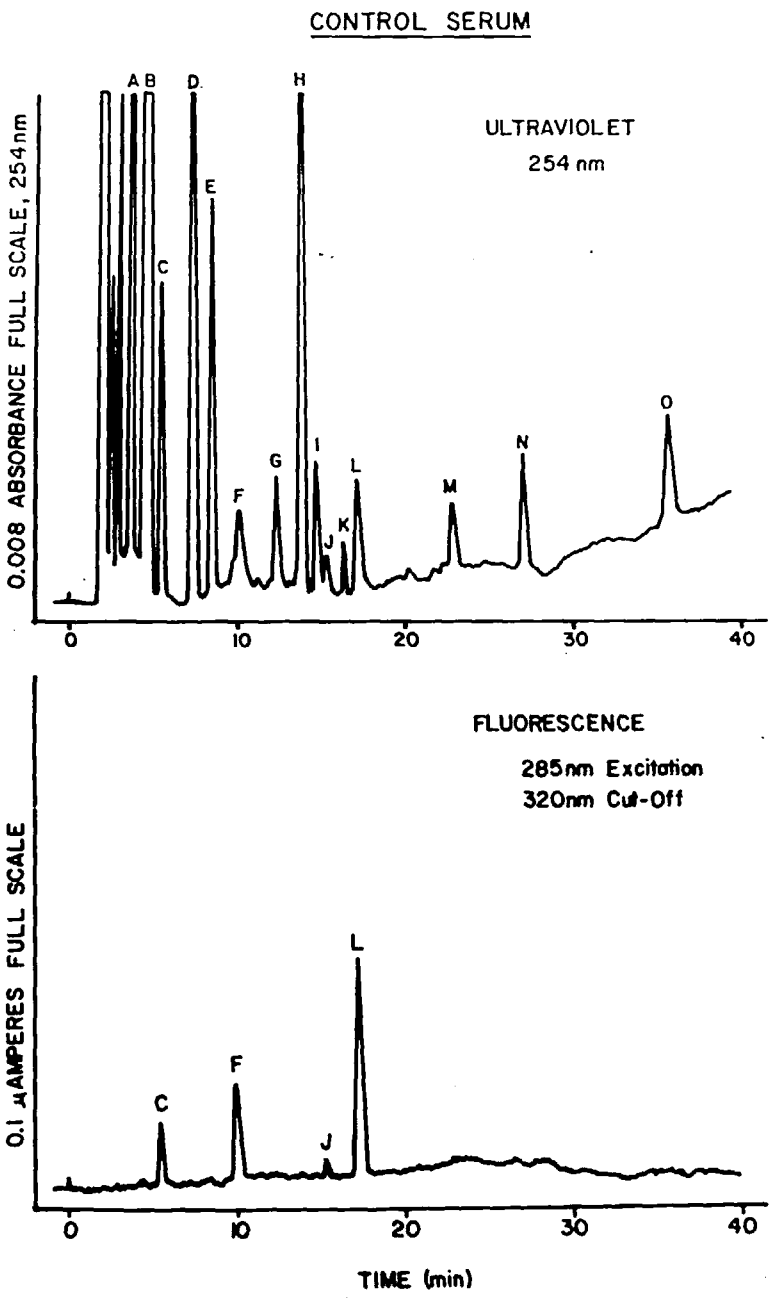

FIGURE 24. Chromatograms of the low molecular weight, UV-absorbing compounds separated in normal human serum under the reversed-phase chromatographic conditions described in Figure 16. The upper chromatogram shows detection at 254 nm, while the lower chromatogram shows the response of a fluorescence detector in series with the UV detector. Sample size: 80 μl of serum ultrafiltrate. Chromatographic conditions: same as those in Figure 16. Fluorescence detector: excitation wavelength, 285 nm, cut-off filter, 320 nm. (Reproduced from Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 186, 647 (1974). With permission.)

terest, yet not so much as to overwhelm the unknown peak so that shoulders or broadening of the solute band, which would indicate slight differences in retention times, would not be detected.

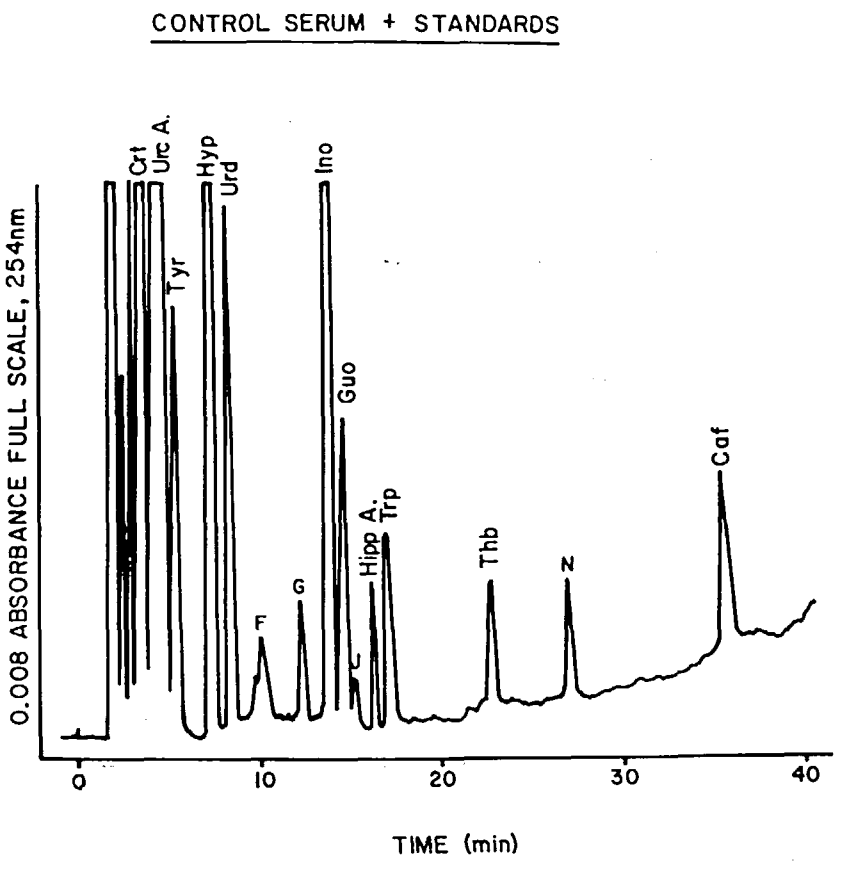

FIGURE 25. The serum sample of Figure 24, but co-injected with the nucleosides, bases, and aromatic amino acids indicated in the figure. Sample and chromatographic data the same as that in Figure 24. (Reproduced from Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 186, 647 (1974). With permission.)

If the separation of the serum sample shown in Figure 24 could be reproduced on a different column or with an entirely different solvent system (pH, organic, gradient, etc.), then correlation of the standards with the serum peaks under all conditions would give fairly positive identification of the solute peak. If one is fortunate enough to find the correct standard, then a match-up of all chromatographic data is possible. However, when dealing with samples of biological origin, often the correct standard is not easily found. Also, it is seldom possible in practice to develop a new separation to test the equivalency of chromatographic behavior between a standard and a solute peak. Thus, methods must be used to insure that a peak with the same retention time as a standard is truly the same compound and not another compound with similar chromatographic properties under the particular conditions used.

B. Spectral Data

1. Absorbance Ratios

The use of a dual wavelength detector or sequential detectors set at different wavelengths can provide a fair amount of spectral information rapidly and inexpensively. The peak height or area ratios obtained represent the ratios of the absorbance values taken at two or more discrete wavelengths. Two compounds which elute with the same retention times will often have different absorbance ratios and can be differentiated on this basis alone.

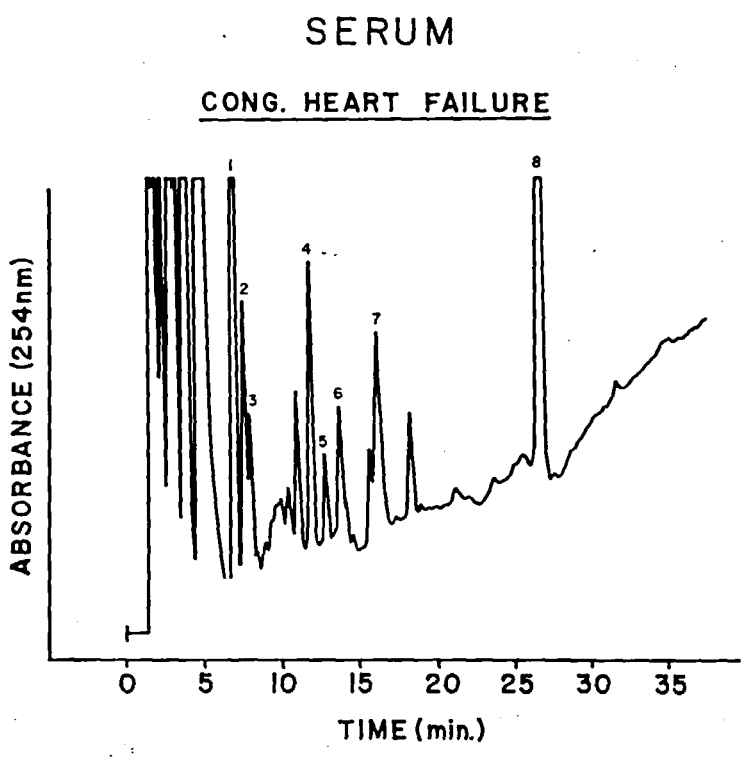

FIGURE 26. Separation of the components in a serum sample from a patient with congestive heart failure. A comparison of the peak height ratios between the serum peaks and co-injected standards is given in Table 10. Sample was prepared using trichloroacetic acid as the protein denaturant. Injection volume: 60 μl; chromatographic conditions: same as those in Figure 16, but with a mobile phase pH of 5.5. (Reprinted with permission from Krstulovic, A. M., Brown, P. R., and Rosie, D. M., *Anal. Chem.*, 49(14), 2237 (1977). Copyright by the American Chemical Society.)

Figure 26 shows a chromatogram of a serum sample processed using TCA. The serum was from a patient with congestive heart failure. The peaks of interest are labelled as 1 to 8. Table 10 presents a comparison of the peak height ratios (254 nm/280 nm) of the serum peaks with standards having the same retention times (from co-injections) as the serum peaks. The excellent agreement and the wide range of peak height ratios observed demonstrate the usefulness of this technique for rapid, on-line confirmation of peak identity and purity. Because small variations in band width and calibrations can cause large differences in the absorbance ratios, it is probably wise to match up standards and unknown peaks on a particular instrument rather than to rely upon literature values.

2. UV Spectra

In cases where the absorbance ratios of questionable peaks are not definitive or where a match with standard reference compounds cannot be made, a full scan of the absorbance spectra can be very useful. Previously it was necessary to collect a peak and use an off-line spectrophotometer to obtain such data. However, there are presently instruments on the market which can produce high-quality absorption spectra on line at sensitivities as high as 0.01 absorbance units full scale (a.u.f.s.).[114]

Figure 27 shows the absorption spectra of several of the peaks from the serum sam-

Table 10
PEAK HEIGHT RATIOS (254 nm/280 nm) FOR STANDARDS AND PEAKS WITH THE SAME RETENTION TIMES IN A SERUM SAMPLE FROM A PATIENT WITH CONGESTIVE HEART FAILURE[a]

Peak no. in serum chromatogram	A 254 nm/A 280 nm (1 SD)	Standards	A 254 nm/A 280 nm (1 SD)
1	19.0 (±0.03)	Hypoxanthine	19.7 (±0.02
2	3.56 (±0.02)	Uridine	3.42 (±0.02)
3	1.78 (±0.03)	Xanthine	1.73 (±0.03)
5	8.41 (±0.04)	Inosine	8.52 (±0.01)
6	2.25 (±0.01)	Guanosine	2.18 (±0.03)
7	0.60 (±0.03)	Tryptophan	0.54 (±0.06)
8	0.83 (±0.03)	1,3-Dimethylxanthine	0.83 (±0.02)

[a] Ratios are average values of triplicate runs.

ple shown in Figure 24. Some of the peaks, such as that identified as hippuric acid and guanosine, have unique spectra that allow for easy identification. The curves shown in Figure 27 were obtained using the stopped-flow technique, whereby the flow is momentarily stopped while the scan is taken.

Because of the high sensitivity, small mismatches between the sample and reference cells are inevitable. Therefore a prior scan of the blank cells is made and is stored in a memory module which is integral with the instrument. Figure 28 shows an uncorrected baseline and the corrected baseline created by the subtraction of the stored background from subsequent spectra. Since most of the solvents used for reversed-phase and ion-exchange separations are not UV absorbing at wavelengths over approximately 220 nm, scans can be made during gradient elution using only the initial background spectrum.

The limitation of using on-line absorbance spectra is the limitation imposed by the chromatographic system itself. Although much information can be obtained by examining the changes in the absorption spectra at different pH values, it is seldom possible to create very acid or alkaline conditions since the mobile phase is the solvent for the peaks. However, if the full pH dependency of individual peaks must be determined, then the peak can be collected and analyzed in a conventional spectrophotometer.

Rapid-scanning spectrophotometers are not yet available commercially. With such instruments, the eluting peaks can be scanned within several milliseconds, thus allowing even a single solute band to be examined throughout, indicating the presence of co-eluting compounds. Limitations of rapid-scanning devices include poor sensitivities and the need for a computer system for data aquisition and storage. The complexity and the cost of such systems will probably approach that of a small GC/MS system, which has similar requirements in terms of data manipulations.

3. Fluorescence

Fluorescence detectors have become available at very moderate cost and can offer high specificity and sensitivity for compounds which have natural fluorescence or which can be reacted to form fluorescent derivatives.

The lower chromatogram of Figure 24 shows the separated serum components detected using a fluorescence monitor. The excitation wavelength was set at 285 nm using a cut-off filter of 320 nm. Very few of the naturally occurring nucleosides exhibit native fluorescence, so that the fluorescent peaks in a sample can be used as negative

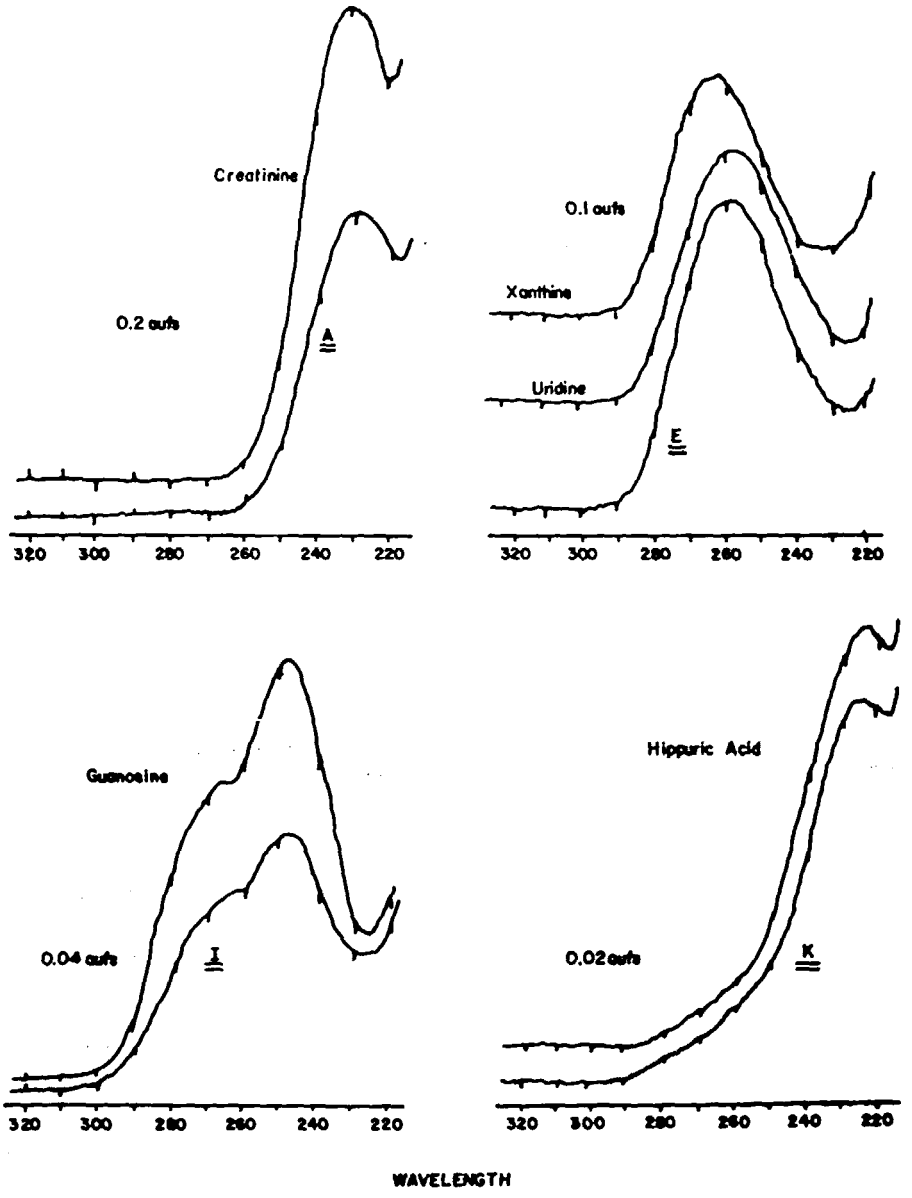

FIGURE 27. A comparison of the UV spectra of standard compounds and the lettered peaks of Figure 24. The spectra were taken by the stop-flow technique, using an on-line UV-Vis spectrophotomer. Chromatographic conditions: same as Figure 16. Sample: the same serum sample as shown in Figure 24.

evidence that they are probably not nucleosides. The exceptions to this are the 7-, 9-disubstituted purines, many of which fluoresce at least weakly.

The strongly fluorescent peak labelled as L in Figure 24 enabled it to be identified as the amino acid L-Tryptophan.[113] The amino acid tyrosine is also readily detectable at the excitation/emission conditions used.

Recent work by Kuttesch et al.[103] has demonstrated the usefulness of derivatization of adenosine to a fluorescent derivative. The reaction of adenosine with chloroacetaldehyde is rapid and complete and enables the development of an assay specific for adenosine, with a lower limit of detection of only 2 pmol.

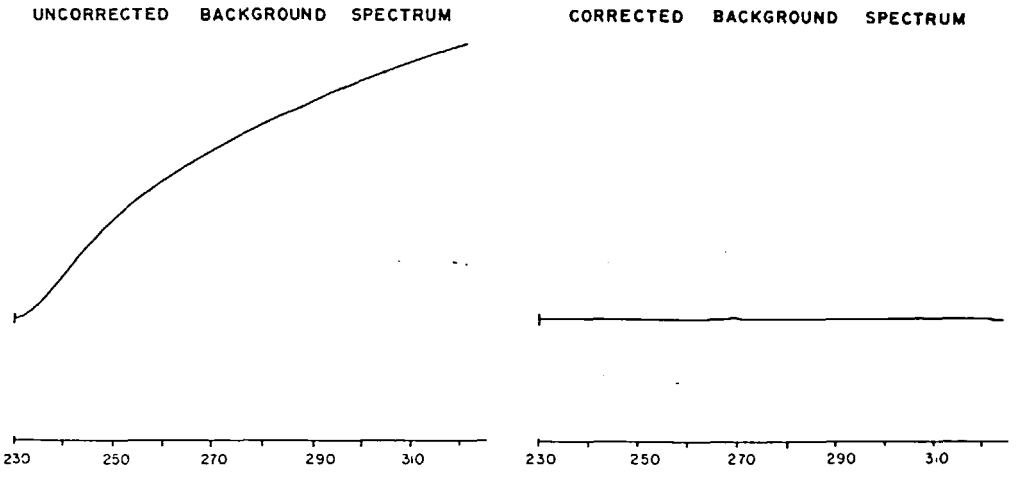

FIGURE 28. The UV absorption curves of the corrected and uncorrected spectra of the UV-Vis spectrophotometer shown in Figure 27. The uncorrected response, due to solvent absorption and spectral differences between cells, is stored in an integral microprocessor memory unit and is subtracted from subsequent spectra. Using this approach, sensitivities as high as 0.01 absorbance units full scale are possible.

Table 11
ENZYMES USEFUL FOR THE IDENTIFICATION OF SOME UV-ABSORBING COMPOUNDS FOUND IN HUMAN SERUM

Substrate(s)	Reagent or cofactor	Enzyme	Reaction conditions	Product(s)
Hypoxanthine	H_2O,O_2	Xanthine oxidase	7.8	Xanthine
Xanthine	H_2O,O_2	(E.C. 1.2.3.2)	7.8	Uric acid
Inosine	Phosphate	Purine nucleoside	7.4	Hypoxanthine
Guanosine	Phosphate	Phosphorylase (E.C. 2.4.2.1)	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

Scanning fluorometers which operate on line with the HPLC are also available[115] and have proven to be quite useful for the identification of specific compounds. When coupled with the wide variety of derivatization reactions available,[116] they can offer a powerful method of peak identification.

C. Enzymatic Peak Shifts

The enzymatic peak shift technique is ideally suited for studies involving biological compounds and can offer a highly specific and sensitive identification of compounds or classes of compounds within a given sample.[109,113] In practice, a small aliquot of sample is reacted with an aliquot of an enzyme solution under the appropriate conditions of pH and temperature. After a suitable period of time, the enzyme is denaturated and the incubated sample is injected into the HPLC. The disappearance of a peak or peaks and/or the appearance of new peaks of the products are evidence that an enzyme-catalyzed reaction has occurred. Table 11 summarizes some enzymes which have proven to be useful for identification of the nucleosides and bases.

Figure 29 shows the identification of the compound adenosine in the plasma extract of a patient suffering from adenosine deaminase deficiency.[76] The removal of the peak labelled adenosine after incubation with adenosine deaminase is strong evidence that the solute is adenosine and not another compound with a similar retention time and absorbance ratio. Less specific enzymes, such as phosphatases, can be useful in identifying certain classes of compounds in a sample, after which identification by retention times and spectral data becomes much easier.

D. Chemical Derivatization

Another method which can yield useful information concerning the presence of certain structures or functional groups is that of chemical derivatization. An aliquot of sample is reacted with an appropriate reagent. The disappearance or appearance of peaks in the chromatogram of the reacted sample as compared to the chromatogram of the original sample is evidence of a chemical reaction. Many of the classical qualitative tests used in organic chemistry as well as the numerous fluorescence and UV-absorbing derivatization reactions[116] are potentially useful. It is possible to identify certain peaks in this way using only microliter quantities of sample.

Figure 30 shows an 80-μl aliquot of the serum sample of Figure 24 after being reacted with sodium periodate. The periodate reaction is highly specific for cis-diols. A close inspection of the two figures will show that the peaks labeled H and I in Figure 24 have been completely removed in Figure 30. Peak E has been considerably reduced in area, while all of the other peaks have remained approximately the same. Peaks H and I had been tentatively identified as inosine and guanosine, while peak E was suspected of containing both xanthine and uridine. The xanthine does not contain a cis diol, but the guanosine, inosine, and uridine all possess the ribosyl moiety. Further work with enzymatic peak shifts, peak height ratios, UV scans, and fluorescence confirmed that peaks H and I were inosine and guanosine, while peak E of Figure 24 consisted of a mixture of both uridine and xanthine, which co-eluted under the chromatographic conditions used.

From this brief summary of some of the methods which can be useful for the identification of compounds of biochemical interest, it is evident that considerable information can be gained rapidly and inexpensively without mass spectral data. A method such as mass spectroscopy is of course indispensible for unambiguous structural determination. However, when a mass spectrometer is not available or when it is poorly suited to the problem at hand, a bit of ingenuity in the application of a combination of techniques can often produce unambiguous identification of the peaks of interest.

V. APPLICATIONS

A. Biochemical Markers

Studies as early as 1957[117] indicated that the urine contained at least several unusual nucleosides and bases.[118] Research quickly followed indicating that the profiles of the excreted nucleosides and bases changed during the course of leukemia[119-123] and other cancers.[124-127] Other researchers have found that the urinary excretion patterns of the nucleosides appear to be altered after X-irradiation damage.[128,129] In addition, some researchers have indicated that the blood levels of adenosine and several of the deoxynucleosides may be increased during the course of myocardial infarction.[130-132]

Borek and Gherke et al. have suggested that the increased urinary excretion of the modified nucleosides in neoplastic patients is a reflection of the increased turnover rate of the tRNA.[134] It is hoped therefore that the urinary concentrations of these compounds might serve as biochemical markers for the presence of certain types of cancer. A recent review by Peng summarizes the biochemistry of the alkylated or otherwise modified nucleosides[133] as well as their role in carcinogenesis.

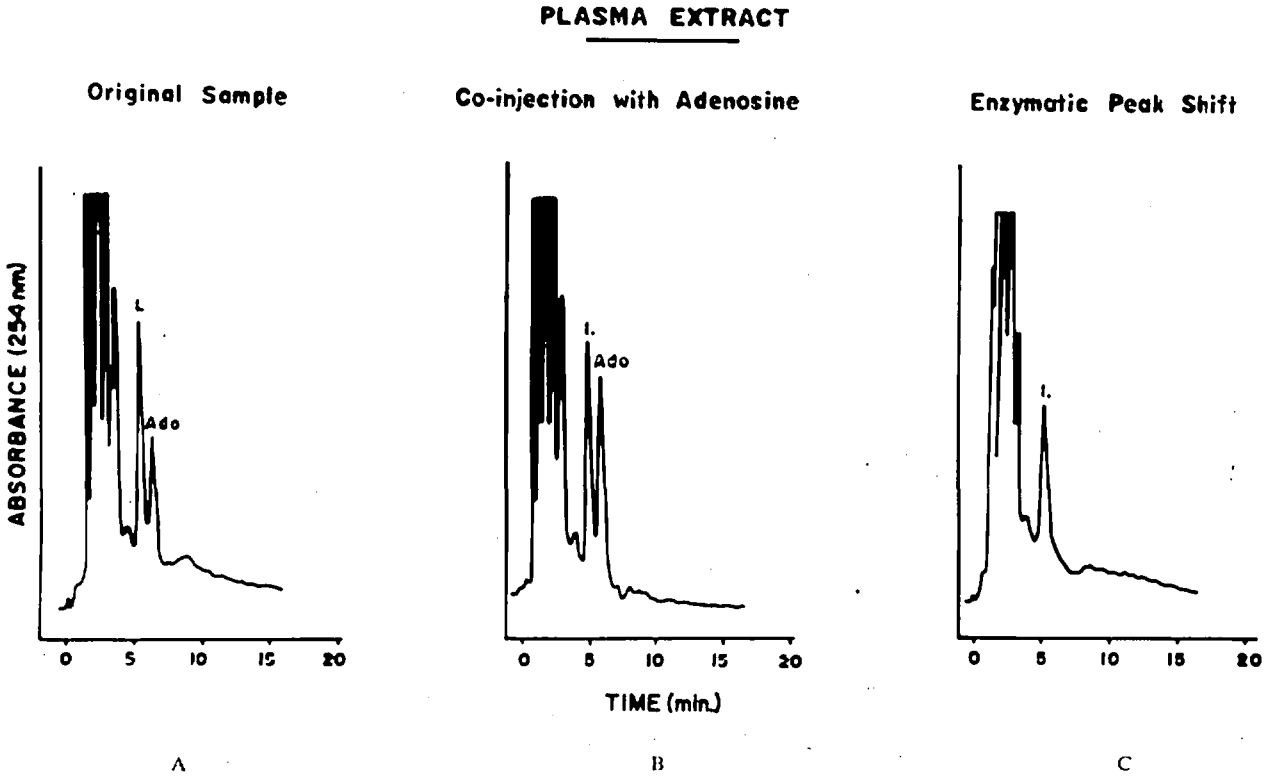

FIGURE 29. The identification of adenosine in the plasma extract of a patient with adenosine deaminase deficiency. Indentified by co-injection and by the enzymatic peak shift method. (A) 50 μl of the plasma extract, (B) 50 μl of plasma extract, co-injected with 50 pmol of adenosine, and (C) 50 μl aliquot of original plasma sample, after incubation with adenosine deaminase. Chromatographic conditions: column: Microbondapak® C_{18}r; eluent: methanol-0.007 mol/l KH_2PO_4, pH 5.8 (10:90, v/v); isocratic elution; temperature: ambient; flow rate: 2.0 ml/min. (Reproduced from Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 142, 383 (1977). With permission.)

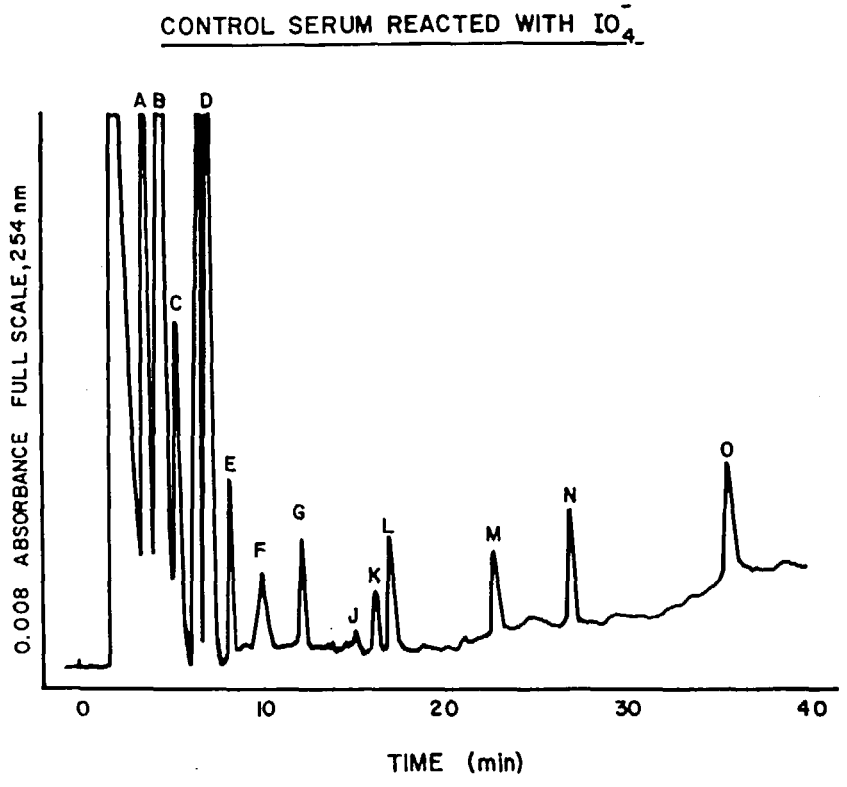

FIGURE 30. Identification of cis-diol moieties in the separated components of the serum sample shown in Figure 24, using periodate oxidation. 80 μl of the serum ultrafiltrate of Figure 24 was incubated with 80 μl of 0.1 mol/l $NaIO_4$ after which the entire 160 μl sample was injected. Peak E was reduced in area by about 50%, while peaks H and I were completely removed. From these and other data, it was concluded that peak E contained both uridine and xanthine, while peaks H and I contained inosine and guanosine, respectively. All conditions identical to those of Figure 24. (Reproduced from Hartwick, R. A., Krstulovc, A. M., and Brown, P. R., *J. Chromatogr.*, 186, 659 (1979). With permission.)

These and other findings have generated considerable interest in the analysis and identification of the nucleosides, bases, and other low molecular weight, UV-absorbing compounds in body fluids and tissues. Although the earliest studies were performed using either GLC with derivatization or open-column (LC) methods, the advent of HPLC enabled the accurate analysis of these compounds in such complex mixtures as urine.

1. Urine

Figure 31 shows the excreted nucleosides in the urine of a patient suffering from colon cancer.[77] The profile of a normal urine sample processed under the same conditions has been presented in Figure 31. A chemically bonded, reversed-phase column and isocratic elution with an acetonitrile buffer eluent was used. The increases in concentration of several of the methylated nucleosides in the colon cancer patient is apparent.

In an earlier study,[135] the 24-hr urinary excretion of several of the methylated nucleosides were studied in over 200 patients with a variety of malignant and nonmalignant diseases, as well as a group of normal control subjects. The scatter diagram in Figure 32 shows that the daily excretion of N_2, N_2-dimethylguanosine was significantly

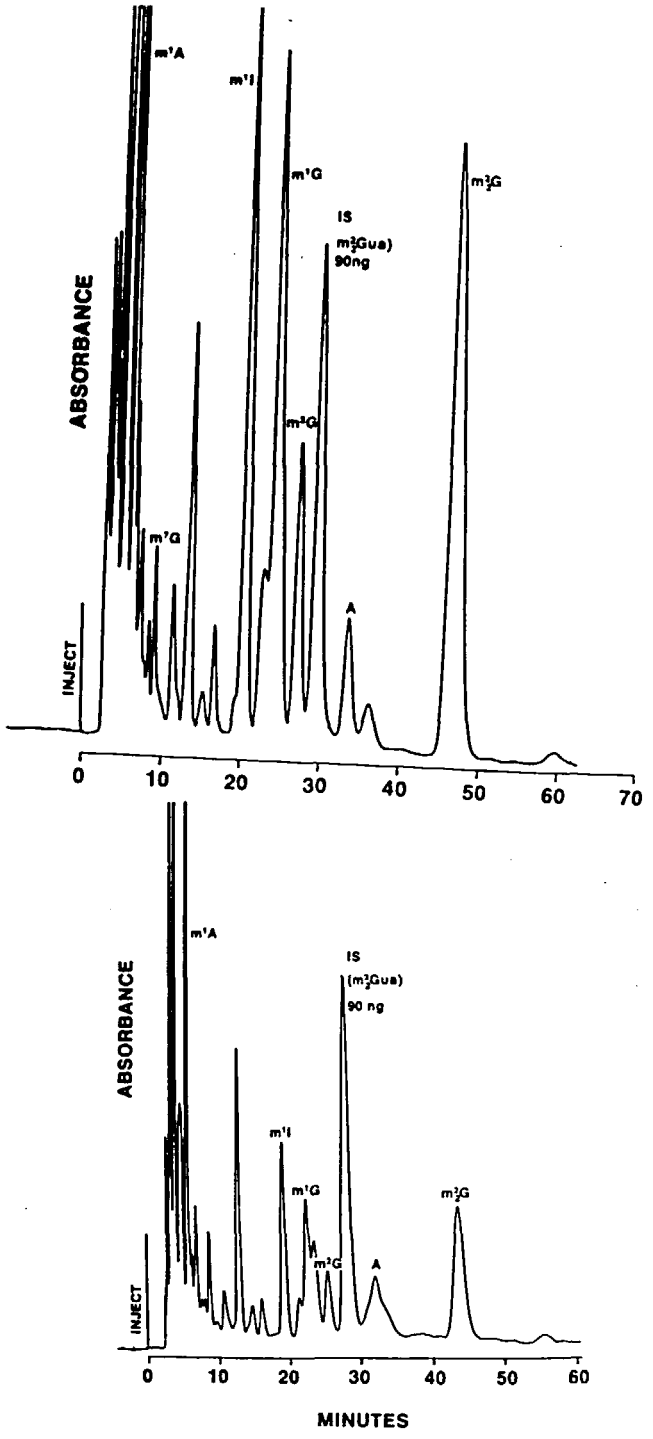

FIGURE 31. The isocratic, reversed-phase separation of the nucleosides isolated in the urine of a patient with colon cancer (upper chromatogram) and a normal control urine (lower figure). Injection volume: 50 μl, equivalent to 25 μl of urine. All other conditions the same as in Figure 13. (Reproduced from Davis, G. E., Suits, R. D., Kuo, K. C., Gehrke, C. W., Waalkes, T. P., and Borek, E., *Clin. Chem. (Winston-Salem, N.C.)*, 23(8), 1427 (1977). With permission.)

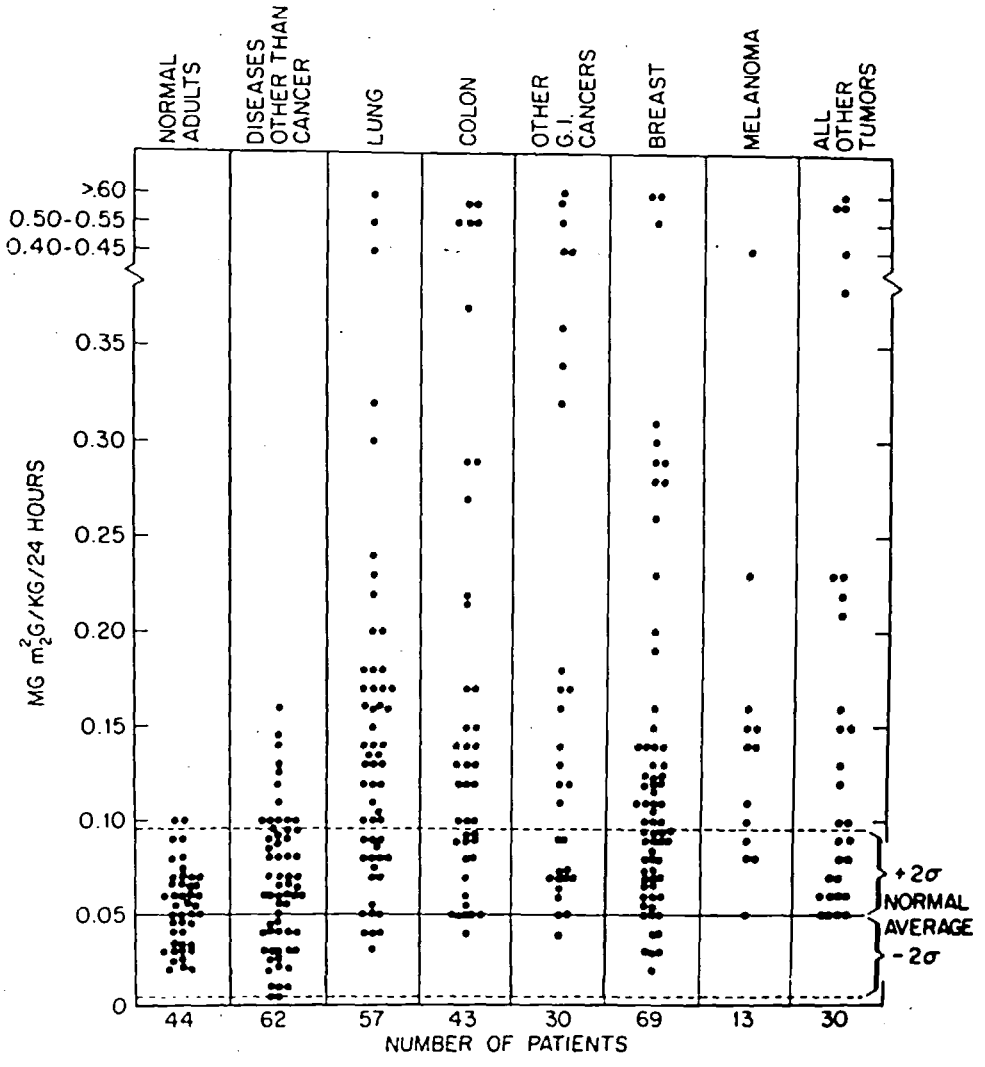

FIGURE 32. Urinary 24-hr excretion values expressed in mg/kg of body weight for normal control adult subjects, for patients with diseases other than cancer, and for patients with cancer. (Reproduced from Tormey, D. C., Waalkes, T. P., Ahmann, D., Gehrke, C. W., Zumwatt, R. W., Snyder, J., and Hansen, H., *Cancer (Brussels)*, 35, 1095 (1975). With permission.)

greater than normal in a large number of the patients suffering from lung, colon, breast, melanoma, and other cancers. The patients suffering from nonmalignant diseases exhibited nearly normal amounts of excreted N_2, N_2-dimethylguanosine in most cases. Similar excretion patterns were observed for the compounds pseudouridine and 1-methylinosine in these same patients.

2. Serum

In serum, some nucleosides and their bases along with a few other UV-absorbing, low molecular weight compounds such as aromatic amino acids, creatinine, and uric acid, can be routinely determined by HPLC.[74,75,147,148] Chromatograms of the constituents in serum are shown in Figure 24. These chromatograms of serum from healthy normal subjects are highly reproducible and showed only slight variations with diet, age, or sex.[148] Table 12 presents a summary of the average serum concentrations for

Table 12
QUANTITATIVE DETERMINATION OF COMPOUNDS IDENTIFIED IN NORMAL HUMAN SERUM

Compound	Sex	$\bar{X}$ (μmol/l)		Range (2 SD)	Literature values* (μmol/l)	Ref.
Creatinine	F	63.4	(8.7)[b]	46.0—80.8	71—106	32
(A)[c]	M	83.1	(11.8)	59.5—107	80—133	32
Uric A (B)	F	171	(30)	110—232	155—357	32
	M	295	(39)	217—373	208—429	32
Tyr (C)	NS[d]	62.2	(16.3)	29.6—94.8	44—71	32
					71—(4)	33
Hyp (D)	NS	7.16	(2.81)	1.56—12.8	3.08—10.9 (male)	36[e]
					1.84—2.5 (female)	
Urd (E)	NS	3.17	(1.11)	0.951—5.39	NA[f]	—
Xan (E)	NS	2.62	(1.04)	0.542—4.70	1.64	—
Ino (H)	NS	5.62	(2.87)	0.0—11.4	NA	—
Guo (I)	NS	0.881	(0.515)	0.0—1.98	NA	—
Hipp A (K)	NS	0.613	(0.477)	0.0—1.57	NA	—
Trp (L)	NS	13.7	(3.57)	6.63—20.8	17—(2)	34
					9.16—(10.4)	35
			Dietary Compounds			
Thb (M)	—	—	—	0.0—6.35	NA	—
Caf (O)	—	—	—	0.0—12.2	NA	—

* All literature values have been converted to micromoles per liter. Standard deviations are given in parentheses. Ranges are indicated by dashes.
[b] Standard deviation.
[c] Letters correspond to the identification of serum peaks given in Figure 24.
[d] Sex related differences were not significant at the 99% confidence level.
[e] Other literature values are presented in this article.
[f] Not available.

14 females and 17 males of the compounds shown in Figure 24. Literature values are given for comparison where available. It can be seen that in most cases, the agreement is excellent between the literature and the experimental (HPLC) values. Peaks were tentatively identified by co-injection of standards (Figure 25), and supportive evidence for their identities was obtained using a combination of methods (see Section IV).

The sera from a number of cancer patients were analyzed in order to determine if alterations in these serum profiles were observable. Figure 33 shows the serum profile of a postoperative patient suffering from malignant breast cancer with metastastasis to the bone. The presence of the peak tentatively identified as 1-methylinosine was unique to the malignant breast carcinoma patients. The scatter diagram of Figure 34 shows the results of this study, which involved a total of 22 patients with histologically documented breast cancer, 8 with benign fibrocystic changes and 10 normal controls. 1-Methylinosine was detected in 45.5% of the breast cancer patients. Similarly, N_2, N_2-dimethylguanosine was found in a total of 22.7% of the sera of the breast cancer patients. Only one false positive was found for 1-methylinosine in the normal group and none for N_2, N_2-dimethylguanosine in either the control or the benign groups.

Other types of cancer were studied in an effort to determine in which types of cancer detectable changes occurred. Figure 35 shows the serum profile of a 46-year-old male patient suffering from lung cancer. When compared with the normal serum profile of Figure 24, two peaks are found which do not occur in normal serum. These peaks have been tentatively identified as 1-methylinosine and N_2, N_2-dimethylguanosine. It

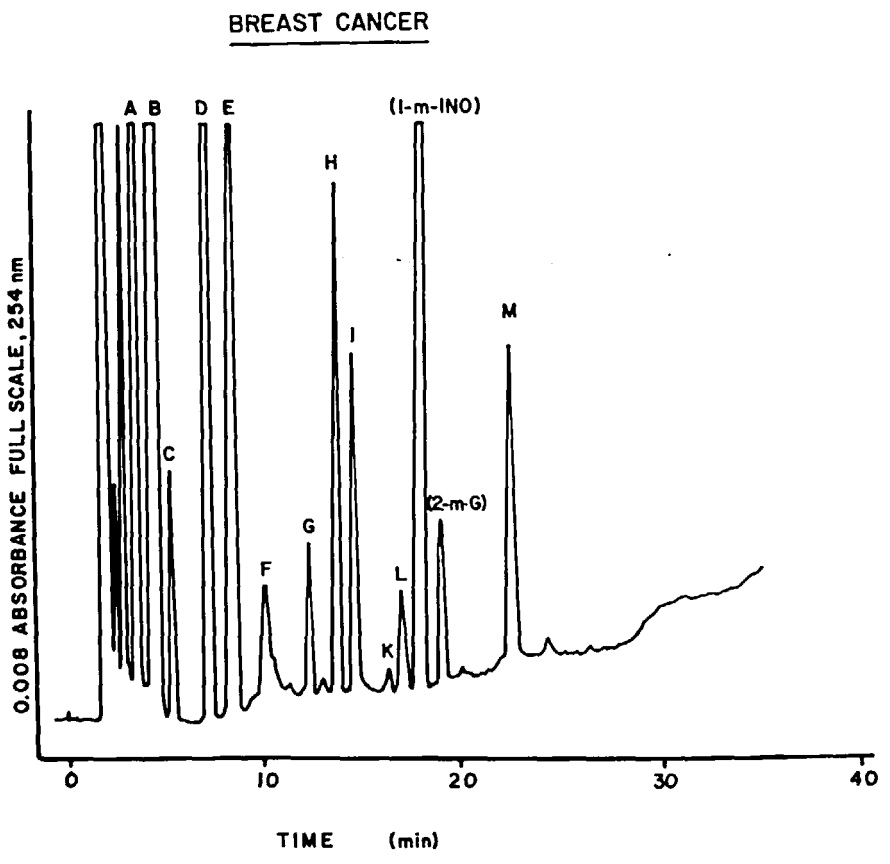

FIGURE 33. Chromatogram of the serum ultrafiltrate of a patient with malignant breast cancer. A normal control subject is shown in Figure 24. All sample and chromatographic conditions the same as Figure 24. (Reproduced from Hartwick, R. A., Krstulovic, A. M., and Brown, P. R., *J. Chromatogr.*, *186*, 659 (1979). With permission.)

is interesting to note that in these preliminary studies within our laboratory, apparently similar changes have been seen in the lung cancer patients and in several heavy smokers. In all, the sera of a total of over 150 cancer patients have been studied to date to determine which types of cancer cause the most pronounced changes in serum profiles. While there is still insufficient data to state that serum nucleoside and base profiles might serve as useful markers for certain types of cancers, it appears that changes definitely occur in the sera of several types of cancer patients. Further work will indicate how sensitive and consistent such changes are to the first stages of neoplastic diseases.

B. Dialysates

A potentially exciting use of the reversed-phase HPLC separations is in the analyses of the dialysate of renal failure patients.[136,137] Using a sodium acetate buffer/methanol gradient, Knudsen et al.[137] were able to separate a wide variety of compounds in the urine, serum, and dialysate of renal failure patients. Figure 35 shows the chromatograms for these separations. This work illustrates the new avenues of research which are being opened by the latest advances in LC technology. Studies such as that presented in Figure 35 will be useful in increasing the clinician's understanding of the basic metabolic processes and the changes which occur in renal failure.

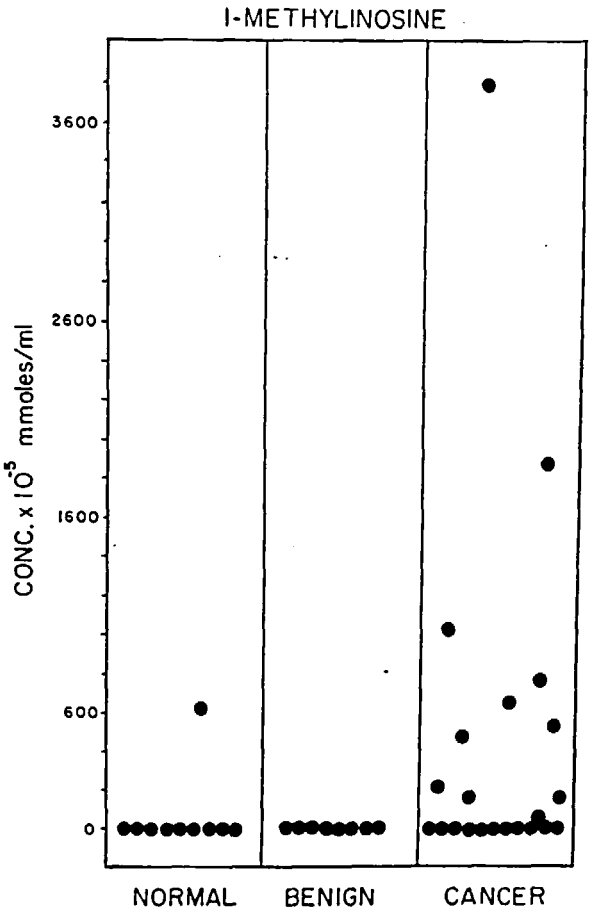

FIGURE 34. Scatter diagram summarizing the serum concentration of 1-methylinosine found in the sera of 10 normal controls, 8 subjects with benign fibrocystic changes, and 22 patients with histologically documented breast cancer. Chromatographic conditions: same as Figure 26.

C. Metabolic Studies

Breter[138] has recently utilized cation-exchange HPLC to study the metabolism of 6-mercaptopurine in cultured cells and animal tissues. The speed and resolving power of HPLC permit the study of an entire system simultaneously, so that the rates of formation of the various metabolites can be accurately studied without using time-consuming multiple analytical techniques. Earlier, Brown[139-141] used anion exchange to study the incorporation of nucleoside analogs into nucleotide pools in humans and for studies of nucleotide metabolism in humans and other species.[141-143]

D. Adenosine

HPLC has been used with great success to study the role of adenosine in various physiological processes.[103] Adenosine plays a critical role in numerous physiological systems. It is believed to play a causal role in severe combined immunodeficiency (SCID), which is characterized by the absence of adenosine deaminase. Figure 36 shows the separation of the $1,N_6$-etheno derivative of adenosine and deoxyadenosine in the extract of a normal urine sample and in the urine extract of a patient suffering

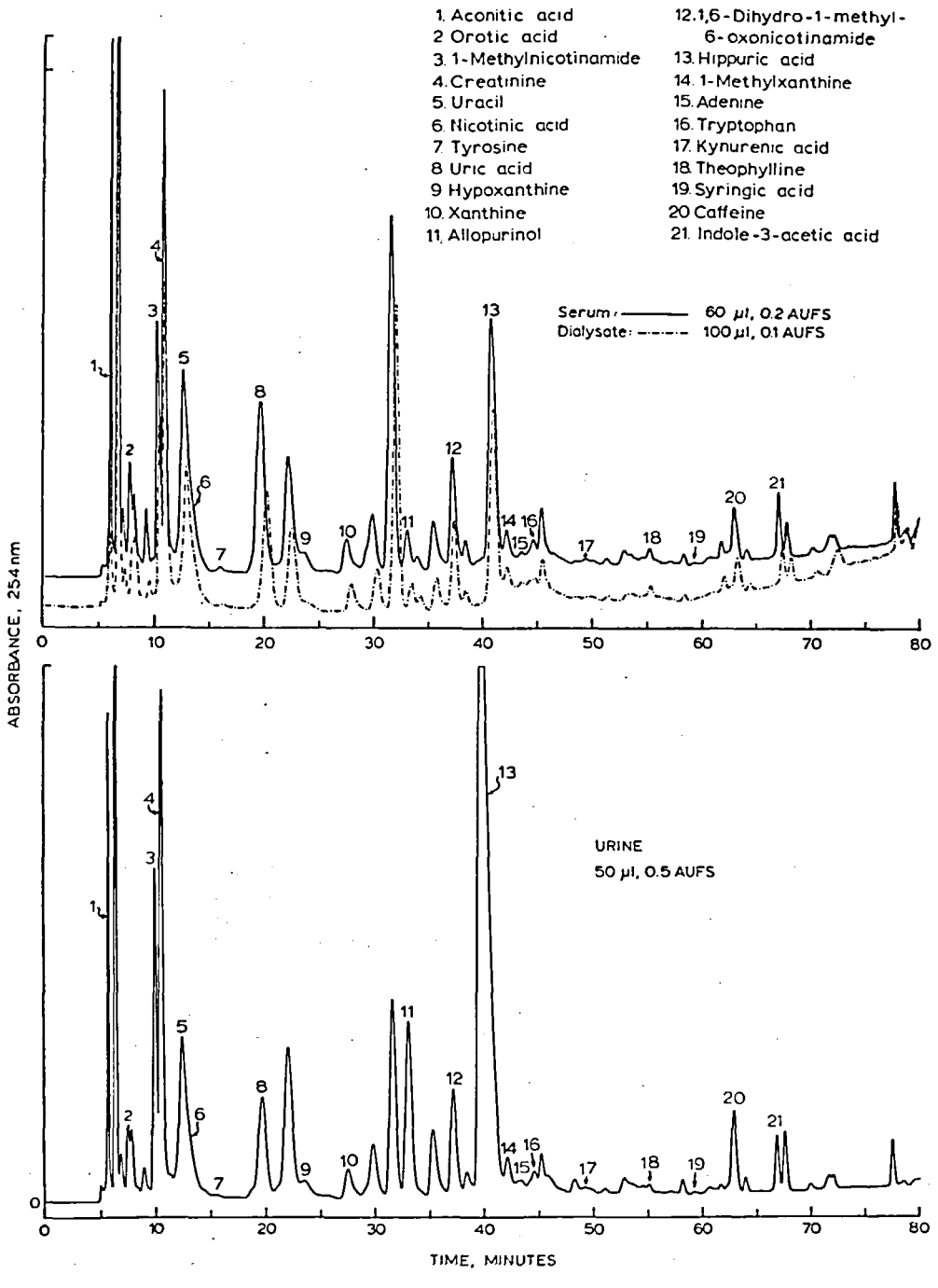

FIGURE 35. Liquid chromatograms for dialysate, serum, and urine samples simultaneously from a uremic patient. Peak identities: 1, aconitic acid; 2, orotic acid; 3, 1-methylnicotinamide; 4, creatinine; 5, uracil; 6, nicotinic acid; 7, tyrosine; 3, uric acid; 9, hypoxanthine; 10, xanthine, 11, allopurinol; 12, 1,6-dihydro-1-methyl-6-oxonicotinamide; 13, hippuric acid; 14, 1-methylxanthine; 15, adenine; 16, tryptophan; 17, kynerenic acid; 18, theophylline; 19, syringic acid; 20, caffeine; 21, indole-3-acetic acid. Chromatographic conditions: gradient elution; low-strength eluent: sodium acetate, 25 mmol/*l*, pH 4.50; high strength eluent: 0.1 mol/*l* acetic acid in methanol; concave gradient; flow rate: 1.0 m*l*/min; detection at 254 nm. (Reproduced from Knudson, E. J., Lau, Y. C., Veening, H., and Dayton, D. A., *Clin. Chem. (Winston-Salem, N.C.)*, 24, 686 (1978). With permission.)

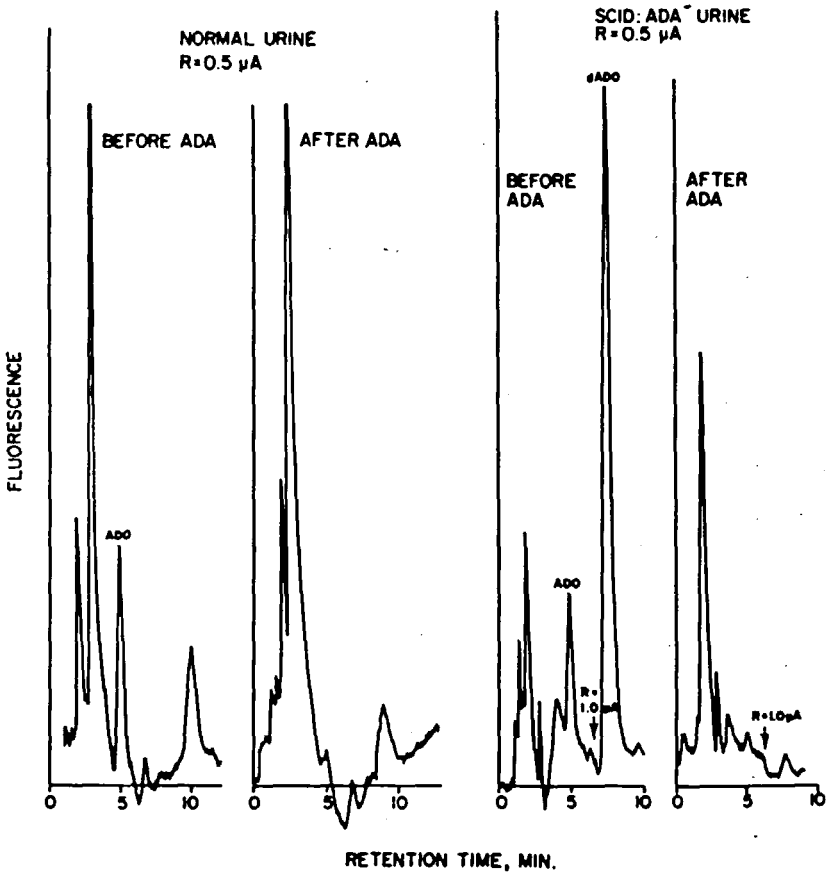

FIGURE 36. Representative chromatograms of human urine illustrating adenine compounds in a normal individual and an SCID:ADA patient. 1-N_6-Etheno derivatization was performed without prior treatment of the extracts with adenosine deaminase (before ADA) and after treatment of the extracts with adenosine deaminase (after ADA). The injection volume (50 μl) was equivalent to 3 μl of urine. Chromatographic conditions: column: Partisil SCX®; isocratic elution using a mobile phase 0.100 mol/l in $NH_4H_2PO_4$, pH 4.5, at a flow rate of 2.0 ml/min. Fluorescence was monitored using an FS-970 detector (Kratos Corporation, Schoeffel Instrument Division), with an excitation wavelength of 280 nm and a cut-off filter of 390 nm. (Reprinted from Kuttesch, J. F., Schmalstieg, F. C., and Nelson, J. A., *J. Liq. Chromatogr.*, 1, 97 (1978) by courtesy of Marcel Dekker, Inc. With permission.)

from SCID. The adenosine and adenine concentrations were found to be elevated in the lymphocytes and erythrocytes, and adenosine in the plasma of SCID patients. These data suggested that there was complete clearance of deoxyadenosine by the kidney and that deoxyadenosine would be detected as deoxyinosine in a normal subject with adenosine deaminase activity.

E. Nucleoside Analogs

Another area of application for HPLC is that of the biochemistry of the nucleosides, antibiotics, and the synthetic nucleoside and base analogs. Plunkett has recently presented an excellent review on the use of HPLC in research on the purine analogs.[145] The metabolism of 9-β-D-arabinofuranosyl-adenine (ara-A) was studied in regards to its incorporation into the nucleotide pools of the body, and Nelson[146] recently studied

the metabolism of 6-thiopurines in *bacillus subtilis*. Such studies are of fundamental significance in the understanding of the biochemistry of such compounds, many of which are highly effective anticancer drugs. As the metabolism of such antimetabolites is better understood, it should become possible to design more effective drugs at the molecular level.

VI. CONCLUSIONS AND FUTURE TRENDS

It is hoped that the discussion presented in this article will bring the researcher up to date on the present status of the HPLC analysis of nucleosides and bases in biological systems. As in many areas of biochemistry, the greatest impact of the most recent advances in HPLC have yet to be felt. Within the last few years, the HPLC methodologies available to the researcher have improved dramatically. Column efficiencies and versatility have been greatly improved. Also, the refinement of on-line identification techniques, such as the UV and fluorescence scanning detectors, has greatly increased the power of the chromatography by obviating the need to collect fractions for further analysis. The sensitivity of the HPLC techniques is usually in the picomole to nanomole range, with a quantitative precision of about 5 to 10% even at this low level.

In the future, other detectors, such as the electrochemical and radioactivity detectors, will be used more widely and the use of a battery of detectors monitoring the effluent for complementary physical or chemical properties will be more commonplace.

Future trends will undoubtedly involve further improvements in column efficiency and reliability. Devices which utilize inexpensive, disposable columns appear promising by reducing the wall effect found in steel columns. The development of both packed and open tubular capillary columns is an area receiving much interest currently. In addition to greatly increasing column efficiencies, such systems may hold the key to the successful mating of liquid chromatography with mass spectometry. The wider use of both LC/MS systems and the introduction of rapid scan techniques will be of great utility for some complex applications, although such systems will bear a heavy burden to the researcher in terms of cost and maintenance.

The use of microprocessors both in the operation of the chromatograph and in data processing and analysis will be required to handle a large volume of samples and the great amount of data generated. Microprocessors are fast becoming an integral part of all analytical instrumentation, and LC, as one of the most recent techniques, has already been improved tremendously by automated gradient, injection and data acquisition systems.

In the area of applications, it remains to be seen whether or not the analysis of urinary or serum nucleosides and bases will have potential for use as biochemical markers. However, the profiles of compounds observed in these fluids will certainly be of great benefit for elucidation of the metabolism and biochemistry of the nucleic acid components in the body.

An area of research which is benefiting from the methodologies discussed here is that of synthesis. In the synthesis of analogs or the isolation of new antibiotics, good use of the powerful resolution and sensitivity of HPLC is being made.[146] When used on the preparative scale, the synthetic chemist can avoid the tedious recrystallizations and less efficient thin-layer chromatography separations of the nucleoside and base analogs and intermediates in their synthesis.

REFERENCES

1. IUPAC, *J. Am. Chem. Soc.*, 82, 5566 (1960).
2. American Chemical Society/Chemical Society, *J. Org. Chem.*, 28(2), 291 (1963).
3. Suhaldolnik, R. J., *Wiley Interscience*, New York, 1970, 128.
4. Pillman, B. and Pullman, A., Electronic Aspects of Purine Tautomerism, in *Advances in Heterocyclic Chemistry*, Vol. 13, Katritzky, A. R. and Boulton, A. J., Eds., Academic Press, New York, 1971, 77.
5. Windholz, M., Budavari, S., Stroumtsos, L. Y., and Fertig, M. N., Eds., *The Merck Index*, 9th ed., Merck and Co., Inc., Rahway, N.J., 1976.
6. Sober, H. A., Ed., *Handbook of Biochemistry*, 2nd ed., CRC Press, Cleveland, 1970.
7. Hall, R. H., *The Modified Nucleosides in Nucleic Acids*, Columbia University Press, New York, 1971.
8. Hotchkiss, R. D., *J. Biol. Chem.*, 175, 315 (1948).
9. Burdon, R. H. and Adams, R. L. P., *Biochim. Biophys. Acta*, 174, 322 (1969).
10. Craddock, V. M., *Biochim. Biophys. Acta*, 240, 376 (1971).
11. Borek, E. and Kerr, S., *Adv. Cancer Res.*, 15, 163 (1972).
12. Mandel, L. R., Srinivasan, P. R., and Borek, E., *Nature (London)*, 209, 586 (1966).
13. Meselson, M., Yuan, R., and Heywood, J., *Annu. Rev. Biochem.*, 41, 447 (1972).
14. Marx, J. L., *Science*, 180, 482 (1973).
15. Pratt, W. B., *Fundamentals of Chemotherapy*, Oxford Press, London, 1973, 10.
16. Knox, J. H., *J. Chromatogr. Sci.*, 15, 333 (1977).
17. Simpson, C. S., *Practical High Performance Liquid Chromatography, Part I*, Heyden and Son Ltd., London, 1976.
18. Karger, B. L., Snyder, L. R., and Horvath, C., *An Introduction to Separation Science*, John Wiley & Sons, New York, 1973, chaps. 5, 10.
19. Giddings, J. C., *The Dynamics of Chromatography, Part I*, Marcel Dekker, New York, 1965.
20. Miller, J. M., *Separation Methods in Chemical Analysis*, John Wiley & Sons, New York, 1975.
21. Brown, P. R., *High Pressure Liquid Chromatography: Biochemical and Biomedical Applications*, Academic Press, New York, 1973.
22. Done, J. N., Knox, J. H., and Loheac, J., *Applications of High-Speed Liquid Chromatography*, John Wiley & Sons, New York, 1974.
23. Scott, R. P. W., *Contemporary Liquid Chromatography*, John Wiley & Sons, New York, 1976.
24. Cohn, W. E., *Science*, 109, 377 (1949).
25. Brown, P. R. and Krstulovic, A. M., Ion-exchange chromatography, in *Techniques of Chemistry*, Vol. 12, 3rd ed., Edmonds, P. and Weissberger, A., Eds., John Wiley & Sons, New York, 1978.
26. Khym, J. X., *Analytical Ion-Exchange Procedures in Chemistry and Biology*, Prentice-Hall, Englewood Cliffs, N. J., 1974.
27. Greene, J. G., Nunley, C. E., and Anderson, N. G., *Nat. Cancer Inst. Monogr.*, 21, 431 (1966).
28. Katz, S. and Burtis, C. A., *J. Chromatogr.*, 40, 270 (1969).
29. Mrochek, J. E., Butts, W. C., Rainey, W. T., and Burtis, C. A., *Clin. Chem.* 17(2), 72 (1971).
30. Singhal, R. P. and Cohn, W. E., *Anal. Biochem.*, 45, 585 (1972).
31. Stahl, K. W., Schlimme, E. and Bojanowski, D., *J. Chromatogr.*, 83, 395 (1973).
32. Brown, P. R., *J. Chromatogr.*, 99, 587 (1974).
33. Lakings, D. B., Waalkes, T. P. and Mrochek, J. E., *J. Chromatogr.*, 116, 83 (1976).
34. Lakings, A. U., Duane, B., Waalkes, T. P., and Mrochek, J., *J. Chromatogr.*, 116, 83 (1976).
35. Floridi, A., Palmerini, C. A., and Fini, C., *J. Chromatogr.*, 138, 203 (1977).
36. Demushki, V. P. and Plyashke, Y. G., Analysis of nucleic acid bases by high pressure liquid ion chromatography, *Anal. Biochem.*, 84(1), 12 (1978).
37. Bakay, B., Nissinen, E., and Sweetman, L., *Anal. Biochem.*, 86, 65 (1978).
38. Eksteen, R., Kraak, J. C., and Linssen, P., *J. Chromatogr.*, 148, 413 (1978).
39. Crampton, C., Frankel, F. R., Benson, P. M., and Wade, A., *Anal. Biochem.*, 1, 249 (1960).
40. Katz, S. and Comb, D. G., *J. Biol. Chem.*, 238, 3065 (1963).
41. Uziel, M., Koh, C. K., and Cohn, W. E., *Anal. Biochem.*, 25, 77 (1968).
42. Murakami, F., Rokushika, S., and Hatano, H., *J. Chromatogr.*, 53, 584 (1970).
43. Breter, H. J. and Zahn, R. K., *Anal. Biochem.*, 54, 346 (1973).
44. Breter, H. J., Seibert, G., and Zahn, R. K., *J. Chromatogr.*, 140, 241 (1977).
45. Essigmann, J. M., Busby, W. F., and Wogen, G. N., *Anal. Biochem.*, 81, 384 (1977).
46. Demushkin, V. P. and Plyashkevich, Yu. G., *Anal. Biochem.*, 84, 12 (1978).
47. Pal, B. C., *J. Chromatogr.*, 148, 545 (1978).
48. Horvath, C. and Lipsky, S. R., *Anal. Chem.*, 41(10), 1227 (1969).

49. Kirkland, J. J., *J. Chromatogr. Sci.*, 10, 129, 1972.
50. Burtis, A. C., Munk, M. N., and MacDonald, F. R., *Clin. Chem. Winston Salem, N.C.*, 16(8), 667 (1970).
51. Henry, R. P., Schmit, J. A., and Williams, R. C., *J. Chromatogr. Sci.*, 11, 358 (1973).
52. Senghal, R. P. and Cohn, W. E., *Biochim. Biophys. Acta*, 262, 565 (1972).
53. Senghal, R. P., *Arch. Biochem. Biophys.*, 152, 800 (1972).
54. Senghal, R. P. and Cohn, W. E., *Biochemistry*, 12, 1532, 1973.
55. Senghal, R. P., *Eur. J. Biochem.*, 43, 245 (1974).
56. Carrara, M. and Bernardi, G., *Biochim. Biophys. Acta*, 155, 1 (1963).
57. Kull, F. J. and Soodak, M., *Anal. Biochem.*, 82, 10 (1969).
58. Hoffman, J. L., *Anal. Biochem.*, 33, 209 (1970).
59. van den Bos, R. C., van Kamp, G. J., and Planta, R. J., *Anal. Biochem.*, 35, 32 (1970).
60. Ehrlich, S., Thieng, J. P., and Bernardi, G., *Biochem. Biophys. Acta*, 246, 161, 1971.
61. Pfadenhauer, E. H., *J. Chromatogr.*, 81, 85 (1973).
62. Khym, J. X., *Anal. Biochem.*, 58(2), 638 (1974).
63. Chen, C. M., Hartnell, G. F., Smith, O. C., and Francis, W. S., *Anal. Biochem.*, 60, 441 (1974).
64. Aoyagi, S., Ishikawa, T., and Genta, H., *Anal. Biochem.*, 65, 567 (1975).
65. Stout, R. W., Chang, C., and Coward, J. K., *Anal. Biochem.*, 76, 342 (1976).
66. Uematsu, T. and Suhadolnik, R. J., *J. Chromatogr.*, 123, 347 (1976).
67. Arendes, J., Zahn, R. K., and Muller, W. E. G., *J. Chromatogr.*, 140, 118 (1977).
68. Goldstein, G., *Anal. Biochem.*, 20, 477 (1967).
69. Kato, Y., Seita, T., Hashimoto, T., and Shimizu, A., *J. Chromatogr.*, 134, 204 (1977).
70. Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 112, 651 (1975).
71. Grushka, E. and Kikta, E. J., *Anal. Chem.*, 49 (12), 1004A (1977).
72. Grushka, E., *Bonded Stationary Phases in Chromatography*, Ann Arbor Science Publishers, Ann Arbor, 1974.
73. Forrest, S. A. and Murphy, R. C., *J. Chromatogr.*, 121, 251 (1976).
74. Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 126, 679 (1976).
75. Brown, P. R., Krstulovic, A. M., and Hartwick, R. A., *J. Clin. Chem. Biochem.*, 14, 282 (1976).
76. Hartwick, R. A. and Brown, P. R., *J. Chromatogr.*, 142, 383 (1977).
77. Davis, G. E., Suits, R. D., Kuo, K. C., Gehrke, C. W., Waalkes, T. P., and Borek, E., *Clin. Chem. Winston Salem, N.C.*, 23(8), 1427 (1977).
78. Brown, P. R., Krstulovic, A. M., and Hartwick, R. A., *Advances in Chromatography*, Vol. 18, Marcel Dekker, New York, in press.
79. Gehrke, C. W., Kuo, K. C., Davis, G. E., Suits, R. D., Waalkes, T. P., and Borek, E., *J. Chromatogr.*, 150, 455 (1978).
80. Scott, R. P. W. and Kucera, P., *J. Chromatogr.*, 142, 213 (1977).
81. Tanaka, N., Goodell, H., and Karger, B. L., *J. Chromatogr.*, 158, 233 (1978).
82. Melander, W., Campbell, D. E., and Horvath, C., *J. Chromatogr.*, 158, 215 (1978).
83. Colin, H., Ward, N., and Guiochon, G., *J. Chromatogr.*, 149, 169 (1978).
84. Colin, H. and Guiochon, G., *J. Chromatogr.*, 158, 183 (1978).
85. Knox, J. H., *J. Chromatogr. Sci.*, 15, 352 (1977).
86. Horvath, C. and Melander, W., *J. Chromatogr. Sci.*, 15, 393 (1977).
87. Persson, B. and Karger, B. L., *J. Chromatogr. Sci.*, 12, 521 (1974).
88. Knox, J. H. and Jurand, J., *J. Chromatogr.*, 125, 39 (1976).
89. Kissinger, P. T., *Anal. Chem.*, 49(6), 883 (1977).
90. Gloor, R. and Johnson, E. L., *J. Chromatogr. Sci.*, 15, 413 (1977).
91. Knox, J. H. and Jurand, J., *J. Chromatogr.*, 149, 297 (1978).
92. Wahlund, K. G. and Beijersten, I., *J. Chromatogr.*, 149, 313 (1978).
93. Hoffman, N. E. and Liao, J. C., *Anal. Chem.*, 49, 2231 (1977).
94. Jandera, P. and Churacek, J., *J. Chromatogr.*, 91, 223 (1974).
95. Jandera, P. and Churacek, J., *J. Chromatogr.*, 91, 207 (1974).
96. Jandera, P. and Churacek, J., *J. Chromatogr.*, 93, 17 (1974).
97. Locke, D. C., *J. Chromatogr. Sci.*, 12, 433 (1974).
98. Liteanu, C. and Gocan, S., *Gradient Liquid Chromatography*, John Wiley & Sons, New York, 1974.
99. Abbott, S. R., Berg, J. R., Achener, P., and Stevenson, R. L., *J. Chromatogr.*, 126, 421 (1976).
100. Schoenmakers, P. J., Billiet, H. A. H., Tijssen, R., and DeGalan, L., *J. Chromatogr.*, 149, 519 (1978).
101. Hartwick, R. A., Grill, C. M., and Brown, P. R., *Anal. Chem.*, 51, 34 (1979).
102. Haftmann, E., *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, D. Van Nostrand, New York, 1975, chap. 2.

103. Kuttesch, J. F., Schmalstieg, F. C., and Nelson, J. A., *J. Liq. Chromatogr.*, 1, 97 (1978).
104. Khym, J. H., *Clin. Chem.*, 21, 1245 (1975).
105. Chen, S. C., Brown, P. R., and Rosie, D. M., *J. Chromatogr. Sci.*, 15, 218 (1977).
106. Van Haverbeke, D. A. and Brown, P. R., *J. Liq. Chromatogr.*, 1, 507 (1978).
107. Hartwick, R. A., Van Haverbeke, D., and Brown, P. R., *J. Liq. Chromatogr.*, 2, 725 (1979).
108. Hartwick, R. A., Jeffries, A., Krstulovic, A. M., and Brown, P. R., *J. Chromatogr. Sci.*, 16, 427 (1978).
109. Krstulovic, A. M., Brown, P. R., Rosie, D. M., and Champlin, P. B., *Clin. Chem. Winston Salem, N.C.*, 23, 1984 (1977).
110. Uziel, M., Smith, L. H., and Taylor, S. A., *Clin. Chem. Winston Salem, N.C.*, 22, 1451 (1976).
111. Boeseken, J., *Adv. Carbohydr. Chem.*, 4, 189 (1949).
112. Opienska, J. B., Charezinski, M., and Brzuszkiewicz, H., *Clin. Chim. Acta*, 8, 260 (1963).
113. Krstulovic, A. M., Brown, P. R., and Rosie, D. M., *Anal. Chem.*, 49(14), 2237 (1977).
114. Krstulovic, A. M., Hartwick, R. A., and Brown, P. R., *J. Chromatogr.*, 158, 365 (1978).
115. Krstulovic, A. M., Powell, A., Hartwick, R. A., and Brown, P. R., *J. Chromatogr.*, 158, 365, 1978.
116. Lawrence, J. F. and Frei, R. W., Chemical derivatization in liquid chromatography, in *Journal of Chromatography Library*, Vol. 7, Elsevier, Amsterdam, 1976, 153.
117. Weissmann, B., Bromberg, P. A., and Gutman, A. B., *J. Biol. Chem.*, 224, 407 (1957).
118. Adler, M., *Science*, 130, 862 (1959).
119. Adams, W. S., Davis, F., and Nakatani, M., *Am. J. Med.*, 28, 726 (1960).
120. Weissman, S. M., Lewis, M., and Karon, M., *Blood*, 22, 657 (1963).
121. Fink, K., Adams, W. S., Davis, F. W., and Nakatani, M., *Cancer Res.*, 23, 1824 (1963).
122. Dlugajczyk, A. and Eiler, J. J., *Proc. Soc. Exp. Biol. Med.*, 123, 453 (1966).
123. Fink, K. and Adams, W. S., *Biochim. Biophys. Acta*, 126, 27 (1968).
124. Chheda, G. B., Mittelman, A., and Grace, J. T., *J. Pharm. Sci.*, 75 (1969).
125. McFarlane, E. and Shaw, G. J., *Can. J. Microbiol.*, 14, 185 (1968).
126. Heirwegh, K. P. M., Ramboer, C., and De Groote, J., *Am. J. Med.*, 42, 913 (1968).
127. Tormey, D. C., Waalkes, T. P., Ahmann, D., Gehrke, C. W., Zumwatt, R. W., Snyder, J., and Hansen, H., *Cancer (Brussels)*, 35, 1095 (1975).
128. Chen, I. W., Kereiakes, J. G., Friedman, B. I., and Saenger, E. L., *Radiology*, 91, 343 (1968).
129. Gurl, C. D. and Cole, L. J., *U.S. Nav. Radiol. Def. Lab.*, 12, 1 (1968).
130. Agostoni, A., Lomanto, B., and Natale, L., *Acta Cardiol.*, 22, 363 (1967).
131. Rubio, R., Berne, R. M., and Katori, M., *Am. J. Physiol.*, 216, 56 (1969).
132. Van Belle, H., *Eur. J. Pharmacol.*, 11, 241, (1970).
133. Peng, A. E., Formation and metabolism of alkylated nucleosides: possible role in carcinogenesis by nitroso compounds and agents, in *Advances in Cancer Research*, Vol. 25, Academic Press, New York, 1977, 195.
134. Borek, E., Baliga, B. S., Gehrke, C. W., Kuo, K. C., Belman, S., Troll, W., and Waalkes, T. P., *Cancer Res.*, 37, 3362 (1977).
135. Waalkes, T. P., Gehrke, C. W., Zumwalt, R. W., Chang, S. Y., Lakings, D. B., Tormey, D. C., Ahmann, D. L., and Moertel, C. G., *Cancer (Brussels)*, 36, 390 (1975).
136. Senftleber, F. C., Halline, A. G., Veening, H., and Dayton, D. A., *Clin. Chem. Winston Salem, N.C.*, 22, 1522 (1976).
137. Knudson, E. J., Lau, Y. C., Veening, H., and Dayton, D. A., *Clin. Chem. Winston Salem, N.C.*, 24, 686 (1978).
138. Breter, H. J., *Anal. Biochem.*, 80, 9 (1977).
139. Brown, P. R., *J. Chromatogr.*, 52, 257 (1970).
140. Brown, P. R. and Parks, R. E., Jr., *Anal. Chem.*, 45, 948 (1975).
141. Parks, R. E., Jr. and Brown, P. R., *Biochemistry*, 12, 3294 (1973).
142. Brown, P. R., Parks, R. E., Jr., and Herod, J., *Clin. Chem. Winston Salem, N.C.*, 19, 919 (1973).
143. Scholar, E. S., Brown, P. R., and Parks, R. E., Jr., *Cancer Res.*, 32, 259 (1972).
144. Senft, A. W., Miech, R. P., Brown, P. R., and Senft, D. C., *J. Int. Parisitol.*, 2, 249 (1972).
145. Plunkett, W., The use of high-pressure liquid chromatography in research on purine nucleoside analogs, in *Adances in Chromatography*, Giddings, J. C., Grushka, E., Kazes, J., and Brown, P. R., Eds., Marcel Dekker, New York, 1978, Chap. 7.
146. Harris, B. A., Weigent, D. A., and Nelson, J. A., *J. Biochem. Pharmacol.*, in press.
147. Hartwick, R. A., Assenga, S., and Brown, P. R., *J. Chromatogr.*, 186, 647 (1979).
148. Hartwick, R. A., Krstulovic, A. M., and Brown, P. R., *J. Chromatogr.*, 186, 659 (1979).