Journal of Chromatography, 379 (1986) 269—311
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2988

REVIEW

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROFILING OF NUCLEIC ACID COMPONENTS IN PHYSIOLOGICAL SAMPLES

RICHARD C. SIMPSON and PHYLLIS R. BROWN*

Department of Chemistry, University of Rhode Island, Kingston, RI 02881 (U.S.A.)

(First received October 24th, 1985; revised manuscript received December 2nd, 1985)

CONTENTS

0378-4347/86/$03.50

1. INTRODUCTION

1.1. Background

The detection and quantitation of nucleotides, nucleosides and bases is becoming increasingly important in the field of biomedical research. These nucleic acid components may be found in physiological fluids, tissues and cells. Their presence is a result of the catabolism of nucleic acids, enzymatic degradation of tissues, dietary habits and various salvage pathways. Since nucleotides, nucleosides or their bases are involved in cellular proliferation and metabolism, altered concentrations of these compounds may be used to indicate the presence of various disease states which cause alterations in the normal purine and pyrimidine metabolic pathways.

Therefore nucleotides, nucleosides or bases may be used as clinical markers for the presence of a disease state. For example, in patients with various forms of cancer, such as leukemia and lung, colon or breast cancer, elevated concentrations of specific nucleic acid components have been found in urine or plasma [1–4]. In one study a mathematical equation was developed to differentiate leukemic from normal populations based upon differences in plasma or serum nucleoside and base concentrations [3,4]. Abnormal nucleoside or base concentrations have also been found to be indicative of various enzyme deficiencies [5,6], gout and renal failure [7], xanthinuria [8] and ischemia [9]. An imbalance of intracellular nucleotide pools also was observed as a result of exposure to chemical toxins [10].

These examples demonstrate the value of monitoring physiological samples for nucleic acid constituents. The success of these studies was made possible by the development of high-performance liquid chromatography (HPLC), which is a powerful technique for the separation and quantitation of such components. Recently there has been an impressive growth of publications reporting the use of HPLC for the metabolic profiling of nucleic acid components in physiolog-

ical samples. HPLC offers a rapid, sensitive and selective method for the determination of nucleotides, nucleosides and bases in a wide variety of sample matrices and has been used for many diverse biochemical studies.

1.2. Sample preparation

Sample preparation is an important part of any chromatographic assay. Many different methods of sample preparation are available, each having specific advantages and disadvantages. For a review of common sample preparation procedures, the reader is referred to another source [11]. Ideally the sample preparation step should be simple and fast, provide good analyte recovery, and avoid sample dilution or contamination. When choosing a sample preparation method for a particular assay, many factors must be considered. The factors include recovery of analytes, co-precipitation of analytes, solubility of analytes, thermal and chemical stability of analytes, and protein-bound versus free analytes.

Due to the wide range in chemical and physical properties of biological matrices and analytes, the specific preparation technique must often be tailored for a specific assay. Even in different assays for the same analytes, there is often no consensus as to which preparation method is best or even correct. Therefore, the current review discusses the type of sample preparation used in each assay.

1.3. Organization

In order to avoid duplicating earlier review articles, this review will cover the literature from the 1979–1980 period through the present. However, no review would be complete without mentioning some of the early pioneering work involved in the HPLC separations of nucleotides, nucleosides and bases. As a result of the efforts of Cohn [12], Uziel et al. [13], Burtis et al. [14], Horváth and Lipsky [15], Kirkland [16], Floridi et al. [17], Singhal and Cohn [18], Brown et al. [19], Hartwick et al. [20], Davis et al. [21] and many others, the use of HPLC for profiling nucleic acid components has been able to advance to the current level.

Previous literature reviews on HPLC profiling of nucleic acid components include topics such as sample processing, chromatographic methodologies, retention mechanisms, peak identification and selected biomedical applications [22–34]. Also, a book has recently been published which provides an in-depth discussion of the use of HPLC in nucleic acid research [11].

Organizing a review article is always a difficult task since various methods of structuring the article can be applied. The present review is organized into major sections corresponding to compound classes (i.e. nucleotides, nucleosides or bases). Each major section is then sub-divided according to the sample matrices in which that group of compounds occurs. Therefore the reader can locate quickly information on and references pertaining to separations of a particular class of nucleic acid constituents in a given physiological sample.

The analyst should keep in mind that there is no "best" method, even for a specific type of analysis. Rather, the assay must be tailored to meet the

TABLE 1

COMPOUND ABBREVIATIONS

Abbreviation	Compound	Abbreviation	Compound
Nucleotides			
ADP	Adenosine 5′-diphosphate	dTTP	2′-Deoxythymidine 5′-triphosphate
AMP	Adenosine 5′-monophosphate	dUMP	2′-Deoxyuridine 5′-monophosphate
ATP	Adenosine 5′-triphosphate	GDP	Guanosine 5′-diphosphate
cAMP	Adenosine 3′,5′-cyclic monophosphate	GMP	Guanosine 5′-monophosphate
cCMP	Cytidine 3′,5′-cyclic monophosphate	GTP	Guanosine-5′-triphosphate
cGMP	Guanosine 3′,5′-cyclic monophosphate	IDP	Inosine 5′-diphosphate
cIMP	Inosine 3′,5′-cyclic monophosphate	IMP	Inosine 5′-monophosphate
CDP	Cytidine 5′-diphosphate	ITP	Inosine 5′-triphosphate
CMP	Cytidine 5′-monophosphate	β-NAD	β-Nicotinamide adenine dinucleotide
CTP	Cytidine 5′-triphosphate	NAD	Nicotinamide adenine dinucleotide
cUMP	Uridine 3′,5′-cyclic monophosphate	NADH	Nicotinamide adenine dinucleotide; reduced form
dADP	2′-Deoxyadenosine 5′-diphosphate		
dAMP	2′-Deoxyadenosine 5′-monophosphate	NADP	Nicotinamide adenine dinucleotide; 3′-phosphate
dATP	2′-Deoxyadenosine 5′-triphosphate		
dCMP	2′-Deoxycytidine 5′-monophosphate	NADPH	Nicotinamide adenine dinucleotide 3′-phosphate; reduced form
dCTP	2′-Deoxycytidine 5′-triphosphate		
dGMP	2′-Deoxyguanosine 5′-monophosphate	TDP	Thymidine 5′-diphosphate
dGTP	2′-Deoxyguanosine 5′-triphosphate	TMP	Thymidine 5′-monophosphate
dm6AMP	2′-Deoxy-6-methyladenosine 5′-monophosphate	UDP	Uridine 5′-diphosphate
		UMP	Uridine 5′-monophosphate
dm5CMP	2′-Deoxy-5-methylcytidine 5′-monophosphate	UTP	Uridine 5′-triphosphate
		XMP	Xanthosine 5′-monophosphate
dTMP	2′-Deoxythymidine 5′-monophosphate		
Nucleosides			
Ado	Adenosine	m1Guo	1-Methylguanosine
Ctd	Cytidine	m2Guo	N^2-Methylguanosine
dAdo	2′-Deoxyadenosine	m7Guo	7-Methylguanosine
dCtd	2′-Deoxycytidine	m2,2Guo	N_2^2-Dimethylguanosine
dGuo	2′-Deoxyguanosine	m1Ino	1-Methylinosine
dIno	2′-Deoxyinosine	m7Ino	7-Methylinosine
dm6Ado	2′-Deoxy-N^6-methyladenosine	m7Xao	7-Methylxanthosine
dThd	2′-Deoxythymidine	ψ	Pseudouridine
Guo	Guanosine	Thd	Thymidine
Ino	Inosine	Urd	Uridine
m6Ado	N^6-Methyladenosine	Xao	Xanthosine
m5Ctd	5-Methylcytidine		
Bases			
Ade	Adenine	m1Gua	1-Methylguanine
Cyt	Cytosine	m2Gua	N^2-Methylguanine
Gua	Guanine	m7Gua	7-Methylguanine
Hyp	Hypoxanthine	m1Xan	1-Methylxanthine
m1Ade	1-Methyladenine	m3Xan	3-Methylxanthine
m3Ade	3-Methyladenine	m9Xan	9-Methylxanthine
m6Ade	N^6-Methyladenine	Thy	Thymine
m7Ade	7-Methyladenine	Ura	Uracil
m5Cyt	5-Methylcytosine	Xan	Xanthine
Miscellaneous			
ADA	Adenosine deaminase	PCA	Perchloric acid
5-AICAR	5-Aminoimidazolocarboxamide riboside	PNPase	Purine nucleoside phosphorylase
		RNA	Ribonucleic acid
Caf	Caffeine	Thb	Theobromine
DNA	Deoxyribonucleic acid	Thp	Theophylline
EDTA	Ethylene diamine tetraacetic acid	tRNA	Transfer ribonucleic acid
HGPRTase	Hypoxanthine-guanine phosphoribosyl transferase	TCA	Trichloroacetic acid
		Tris	Tris(hydroxymethyl)aminomethane
IPA	Indole-3-propionic acid	Tyr	L-Tyrosine
Kyn A	Kynurenic acid		
Ord	Orotidine		

requirements for the specific analyte(s) in a particular sample, and the required sensitivity, specificity, etc. Indeed an acceptable method for one analyst may prove to be totally unacceptable, for a variety of reasons, to another. This review will not present the authors' personal opinions nor attempt to single out any method as being the "best" or "only" one to use for a given application. However, advantages and/or disadvantages of the various methods will be presented.

Therefore in this review we have condensed currently available methodologies, summarizing the techniques, advantages, disadvantages, and applications of each method. The final evaluation and selection of an appropriate HPLC method must be made by each investigator for a particular assay.

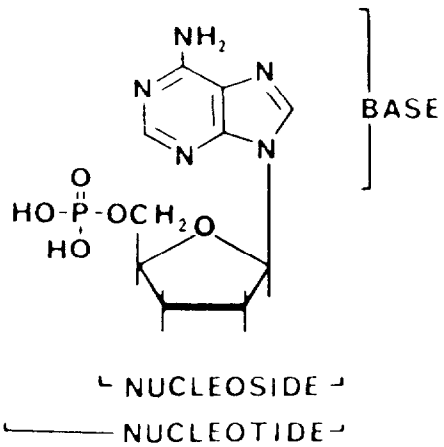
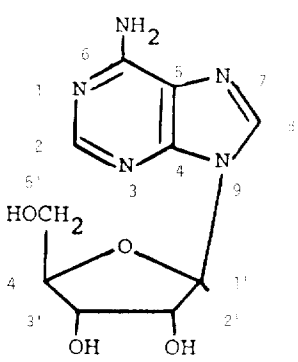


Fig. 1. Structural relationship of nucleotide, nucleoside and base.



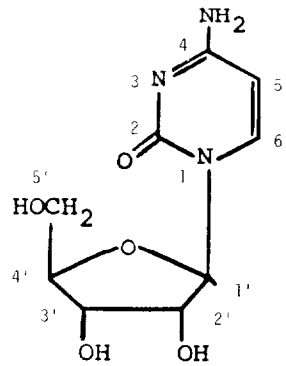


Fig. 2. Systematic numbering of a purine nucleoside (adenosine).

Fig. 3. Systematic numbering of a pyrimidine nucleoside (cytidine).

Table 1 gives a listing of the abbreviations used in this review. Fig. 1 presents the structural relationship of a nucleotide, nucleoside and base. Figs. 2 and 3 show the systematic numbering schemes of purine and pyrimidine nucleosides, respectively.

2. NUCLEOTIDES IN VARIOUS MATRICES

Nucleotides are the major building blocks of nucleic acids, and are therefore important in the transmission of genetic information. Additionally, nucleotides are important in the transfer of chemical energy during cellular metabolism. Therefore researchers have been able to use HPLC profiles of nucleotides in a wide variety of applications.

For HPLC nucleotide assays the analytes must first be extracted from the sample matrix, followed by a high-resolution separation of the nucleotides of interest. Ribonucleotides, especially the triphosphates, tend to break down, particularly in acidic media. Thus the extraction process must be rapid and is generally performed at low temperatures. In the case of a tissue sample, the tissue is usually frozen in liquid nitrogen prior to extraction to decrease the amount of nucleotide decomposition.

When deoxyribonucleotides are the analytes, special procedures such as periodate oxidation or on-line sample clean-up are required to remove the corresponding ribonucleotides which are present in relatively large amounts. This precaution prevents the ribonucleotides from masking the presence of the corresponding deoxyribonucleotides.

2.1. Standard mixtures

In methods development papers, separations of standard mixtures of compounds are often reported. Although the final test of a separation method is its applicability to actual biological samples, separations of standards alone do provide a starting point for biomedical applications. Therefore, separations of nucleotide standard mixtures will briefly be reviewed.

2.1.1. Weak anion exchange

Pon and Ogilvie [35] have reported the use of a silica-based weak anion-exchange column for the simultaneous separation of eight ribonucleotides and ribonucleosides (Ado, Guo, Urd, Ctd and their monophosphates) in enzymatic digests. The separation required a ternary gradient and was completed in approximately 20 min. All eight components were baseline resolved except Ado and Guo, which coeluted. The authors also gave the isocratic conditions for the separation of the four nucleosides and the pH gradient parameters for the separation of the four nucleotides.

2.1.2. Strong anion exchange

2.1.2.1. Synthetic resin. Axelson et al. [36] have used a synthetic resin-based anion-exchange material for the isocratic separation of radioactively labeled and non-labeled nucleoside mono-, di- and triphosphates, including cGMP, cAMP and NAD. The mobile phase contained volatile trifluoroacetic

acid, enabling easy removal of the mobile phase from collected fractions. The method resulted in good recovery (>90%) of the compounds investigated.

2.1.2.2. Inductively coupled plasma detection systems. Two recent publications have used inductively coupled plasma (ICP) as a detection system for phosphorus contained in nucleotides. Heine et al. [37] used a 5-min linear gradient for the anion-exchange separation of selected nucleotides. A total of six nucleotides were separated; however, only three were present in any given standard mixture. The authors found the ICP detection to be less sensitive than UV detection at 254 nm. However, the main advantage of the ICP detector is its high selectivity.

A similar system was also described by Yoshida et al. [38]. Their system used a 2-h gradient for the ion-exchange separation and ICP detection of 12 nucleoside mono-, di- and triphosphates. As in the previous paper, the ICP detector was found to be less sensitive than a UV detection system. However, the article reported that a single calibration for phosphorus was all that was required for the quantitation of all nucleotides, in contrast to the necessity of using a separate calibration curve for each nucleotide with UV detection.

2.1.3. Metal chelate affinity chromatography

Hubert and Porath [39] have used metal chelate affinity chromatography to separate purine mononucleotides from pyrimidine mononucleotides. The column contained a copper-loaded packing. Due to complexation with copper, the purine mononucleotides were significantly retained on the column. Although the method appears limited, it may prove useful for the group separation of various nucleotides.

2.1.4. Zwitterion-pair chromatography

A variation of ion-pair chromatography which employs a zwitterion-pairing agent has been used by Knox and Jurand [40] to separate nucleotides. The zwitterionic pairing agent was 11-aminoundecanoic acid and formed a quadrupolar ion pair with the nucleotide phosphate group. Separations were optimized by adjusting parameters such as pH and pairing agent concentration. The method has been successfully used for the separation of a 16-component mixture of nucleotides, nucleosides and bases.

2.1.5. Boronic acid-substituted silica

A boronic acid-substituted silica support has been used by Glad et al. [41] for the separation of nucleotides, nucleosides and carbohydrates. This type of support has been shown to provide faster, more efficient separations of diol-containing compounds than is possible on polymeric supports such as cellulose and polymethacrylic acid. It is also more selective than some conventional HPLC packing materials.

2.2. Tissues

2.2.1. Cardiac tissue

Due to their importance in cellular energy utilization, nucleotides have been

monitored in cardiac tissue before and after cellular ischemic injury and/or death. Changes in the nucleotide profile can provide insight to the metabolic pathways of cardiac tissues.

In an investigation of cardiac energy metabolism, Juengling and Kammermeier [42] have used ion-pair reversed-phase HPLC for the separation of adenine nucleotides. The compounds separated consisted of a mixture of mono-, di- and triphosphates, plus NAD. The emphasis of the method was on the adenine nucleotides. The separation of AMP, ADP and ATP was complete. However, other peaks eluted very close to AMP and ADP and could potentially cause difficulties in accurate quantitation of AMP and ADP if the nucleotides of other purine or pyrimidine bases were present. The separation was isocratic and rapid (8 min) with 98% recovery of the adenine nucleotides. Sensitivity was on the order of 1 nmol. Sample preparation was accomplished by grinding 300 mg of frozen tissue followed by an extraction of the nucleotides with perchloric acid (PCA).

Harmsen et al. [43] have employed an anion-exchange gradient system for the separation of myocardial adenine nucleotides, creatine phosphate and NAD. Approximately 500 mg of rat cardiac tissue were required for the assay. Sample preparation consisted of grinding the frozen tissue, extraction of nucleotides with PCA and neutralization of the extract. Approximately 28 min were required for the chromatographic separation of all 13 compounds in the standard mixture, which consisted of the mono-, di- and triphosphates of Ado, Ino, Guo, and Urd, plus creatine, creatine phosphate and NAD. The minimum detection limit for AMP was approximately 20 pmol, with a linear range up to 400 nmol. Recovery of AMP was above 95%. The gradient separation provided both better sensitivity and resolution than the previous method [42]. However, the resolution of AMP and NAD was still very difficult. Additionally, the time required for the method was significantly longer than other reported separations. The current method was applied to a study which monitored the effects of anoxia on rat cardiac tissue. The results showed anoxia caused a decrease of ATP and creatine phosphate, along with an increase of AMP. The level of ADP was found to remain essentially unchanged.

Cardiac adenine nucleotide content has also been investigated by Ingebretsen et al. [44]. By use of an isocratic reversed-phase ion-pairing system, separation of AMP, ADP, ATP and Ino was accomplished in 16 min. Standard PCA extraction of frozen tissue was used in the sample preparation. However, only 30 mg (wet weight) of tissue were required, which is significantly less tissue than required in the previous two methods [42,43]. The current method provided excellent resolution of the nucleotides of interest. Additionally Ino, which is produced as a result of AMP degradation during ischemia, was also well resolved and easily monitored.

Another method for monitoring cardiac adenine nucleotides has been described by Hull-Ryde et al. [45]. The authors used a C_{18} radially compressed column to monitor changes in ATP, ADP, AMP, Hyp, Ino and Xan caused by ischemia. Approximately 75–150 mg (wet weight) of tissue were required for the PCA extraction. This weight is also substantially less than that required in previous methods [42,43]. Isocratic separation of all compounds was achieved in 13 min. All components were easily resolved, even in actual sample

matrices. The authors stated that Ado was not monitored in order to avoid use of a gradient. The analysis of Ado, which is generated from AMP degradation, is not important since it quickly diffuses across cell membranes. In addition Ado is rapidly deaminated by adenosine deaminase which is present in large quantities in the myocardium. The resulting Ino could be readily monitored in this system. The results of the study showed a progressive depletion of adenine nucleotides, coupled with an increase in metabolites, as a result of ischemia.

2.2.2. Liver tissue

A publication by Edelson et al. [46] described the ion-exchange separation of various nucleic acid constituents. Although the paper presented mainly methods development data, a separation of the components in mouse liver was presented as an actual application.

The pyrimidine ribonucleotide and deoxyribonucleotide content of mouse liver and cultured mouse lymphoma cells has been studied by Maybaum et al. [47]. Gradient anion-exchange HPLC was used to isolate initially pairs of ribo- and deoxyribonucleotides from neutralized PCA extracts of tissue or cell samples. The separation required 2–2.5 h for completion. The pairs were collected and hydrolyzed with acid phosphatase to yield the corresponding ribo- and deoxyribonucleosides, which were subjected to a 12-min isocratic reversed-phase separation for quantitation. Sensitivity in the reversed-phase mode was found to be approximately 10 pmol. Due to interferences in the reversed-phase separation, quantitation of dCtd was performed on an analytical anion-exchange column. Although the method was time consuming, the authors claimed it was capable of the simultaneous determination of any or all endogenous nucleotides or nucleotide drug derivatives without interferences from the sample matrix.

Riss et al. [48] have studied the nucleotide content of rat liver and hepatocytes by use of anion-exchange HPLC. The sample preparation consisted of a PCA extraction of frozen tissue. An interesting step in the procedure was the actual grinding technique used on the liver tissue. Instead of using a pre-cooled mortar to grind the frozen tissue prior to extraction, the frozen tissue was actually ground under liquid nitrogen. Such a technique helps to slow decomposition of the nucleotides and may provide a more realistic profile of the sample. The separation utilized a combination of flow and salt gradients. The separation of 18 nucleotides required approximately 55 min. Although the system separated some nucleoside 5′-monophosphates from nucleoside 2′- or 3′-monophosphates, several other compounds did coelute. For example, NAD and AMP coeluted. This coelution is a serious disadvantage and prevents measurement of either compound, since both are present in tissue cells. Additionally, guanosine 2′- and 3′-monophosphate were found to coelute with GMP. The paper also included a study of the efficiencies of various extraction procedures. The most satisfactory extraction was a PCA extraction of the tissue, followed by neutralization and extraction of PCA with a freon–amine mixture.

Darwish and Prichard [49] have used a reversed-phase ion-pair technique for the separation of ribonucleotides in sheep liver. The authors developed both gradient and isocratic methods utilizing a radially compressed C_{18} column. The

paper was concerned with 16 nucleotides, especially those of Ade, Gua and Hyp. Trichloroacetic acid (TCA) extraction was used to isolate the nucleotides from the tissue. An isocratic separation was found to give good resolution of the nucleoside triphosphates, as well as some cyclic nucleotides, in 18 min. Use of a gradient provided better resolution of the mono- and diphosphates but required 28 min for completion. The dual-separation approach is attractive if only a specific class of nucleotides (i.e. mono-, di- or triphosphate) is the analyte of interest. However, this approach is not desirable when an overall general profile is the goal, since two separate injections must be made to obtain the total profile.

The nucleotide content of rat liver Golgi vesicles has been determined by a salt and pH gradient anion-exchange method reported by Fleischer [50]. Approximately 30 min were required to separate the nucleotides in a TCA extract of the tissues. A freon–amine mixture was used to neutralize the extract prior to injection. The study was intended to examine the nucleotide metabolism and transport in Golgi cells. Nucleotides found included CMP, UMP, GMP, UDP, ADP and GDP. The results indicated that Golgi cell membranes are not freely permeable to all nucleotides.

Reiss et al. [51] have used radially compressed anion-exchange columns for the separation of up to 20 nucleotides in rat liver and isolated hepatocytes. The separation utilized a salt gradient and was complete in approximately 25 min. To minimize baseline drift due to UV-absorbing impurities in the phosphate buffer, an ion-exchange clean-up procedure was developed to remove the impurities and give a smoother baseline which resulted in improved sensitivity. Use of a radially compressed column allowed a three-fold reduction in separation time over that obtained on conventional steel columns by Hartwick and Brown [52]. Only 10 μg of liver tissue were required for the method, with nucleotide recoveries ranging from 87 to 107%. One disadvantage of the current method was interferences with GDP and UDP, resulting in inability to quantify these two compounds.

2.2.3. Brain tissue

Krstulovic et al. [53] have used reversed-phase HPLC for the separation of the five naturally occurring cyclic ribonucleotides and AMP in a rat brain sample matrix. The brain tissue was extracted with PCA, with the extracts stable for at least five weeks when frozen. Use of a linear methanol gradient provided essentially baseline resolution of all six components in approximately 25 min. A rapid isocratic separation for cAMP in 12 min was also reported. Advantages of the method include sensitivity (50 pmol) and no sample preconcentration requirements. Although applied to brain tissue, the method was also found to be useful for other types of samples such as cell extracts and physiological fluids.

Nucleotide concentrations in ischemic gerbil brain tissue have been quantified by Morimoto et al. [54]. By employment of an isocratic anion-exchange system, ATP, ADP and AMP were separated in 10 min. Sample treatment included in situ freezing of the brain tissue, followed by extraction with PCA. The acidic extract was injected directly. Peak shapes of all three nucleotides have shoulders which indicate that other nucleotides and/or impurities may

have coeluted with the analytes, although there was no discussion of this problem in the paper. Results of the study showed that brain concentrations of ATP decreased while ADP and AMP levels increased as a result of ischemia.

Yamamoto et al. [55] have determined the Gua nucleotide content in rat brain, utilizing reversed-phase ion-pair HPLC and electrochemical detection (oxidative mode). The rats were killed with microwave radiation. Tissue from various regions of the brain was extracted with PCA. The extract was then neutralized and theophylline added as an internal standard. The isocratic separation of GTP, GDP, GMP and cGMP was accomplished in 20 min. The linearity of the method ranged from 0.5 pmol to 1 nmol. Advantages of the system are the high sensitivity and selectivity of the electrochemical detector in response to the electrochemically active Gua nucleotides. Results of the study indicated that GTP was the predominant Gua nucleotide in rat brain tissue, with GDP and GMP present in lesser amounts.

2.2.4. Muscle tissue

Currie et al. [56] have used anion-exchange HPLC for a study involving ATP metabolites in beef skeletal muscle. Sample tissue was frozen in liquid nitrogen and extracted with PCA, which was then neutralized with a freon–amine mixture. The aqueous layer was then injected. Although larger quantities of sample (1 g) and extractant (10 ml) were used in this study, the procedure could easily have been used on a much smaller scale if sample availability was limited. The salt and pH gradient completed the separation of AMP, ADP, ATP, IMP, IDP, ITP, Ino and Hyp in 35 min. The linearity of the method was from 1 nmol to 15 nmol. Several points should be considered when evaluating this method. Hyp and Ino were not completely resolved. Also IDP and ADP were not completely resolved, but were individually quantified. UV-absorbing impurities in the phosphate buffer were removed to eliminate masking of IDP. The removal was accomplished by an ion-exchange clean-up of the buffer prior to use. Any residual impurities in the column were removed with an EDTA wash. Finally, incomplete removal of perchlorate ion from the sample solution resulted in a splitting of the IMP peak. Use of a freon–amine mixture to remove perchlorate from the sample solution generally eliminated this problem.

A method utilizing reversed-phase ion-pair HPLC for use in separating nucleotides in fish muscle has been reported by Murray and Thomson [57]. Tissue was extracted with PCA. The extract was neutralized prior to injection. The system permitted the isocratic separation of Hyp, IMP, Ino, AMP, ADP and ATP in less than 10 min. The linearity ranged from 0.2 to 2.5 nmol.

2.2.5. Skin tissue

Pruneau et al. [58] have employed an anion-exchange separation to monitor ATP and ADP in healing proliferative skin tissue. In addition to ATP and ADP, the pH–salt gradient also separated 13 other nucleotides in 80 min. Sensitivity of the method was 0.25 nmol, with a linear detector response up to 8 nmol. Sample preparation consisted of grinding tissue under liquid nitrogen instead of simply using a pre-cooled mortar. The rapid freezing decreases the breakdown of ATP and ADP. Also, a double PCA extraction was performed on the frozen tissue to maximize nucleotide recovery. The method reports a 97% recovery of

ATP and ADP from a 30-mg skin sample. With such a small sample size, this method may be used for tissue biopsy applications.

2.2.6. Embryo tissue

A method utilizing anion-exchange HPLC has been used by Ritter and Bruce [59] to determine the deoxyribonucleotide content of rat embryonic tissue. Using a pH—salt gradient, the method separated dCTP, dTTP, dATP and dGTP in 105 min. The tissue was frozen on dry ice, followed by a double PCA extraction. The neutralized extract was treated with periodate to convert ribonucleoside triphosphates to their respective bases, leaving only deoxyribonucleoside triphosphates intact for the assay. The method required destruction of the ribonucleoside triphosphates since their concentrations are so high they can mask the presence of any deoxyribonucleoside triphosphates present. A disadvantage of the method was the length of time required to freeze the tissue with dry ice. This slow step may cause concentrations of some deoxyribonucleoside diphosphates observed to be falsely high. Use of liquid nitrogen to freeze the tissue should prove much faster and minimize sample decomposition.

2.2.7. Miscellaneous tissues

Shaw et al. [60] have used weak anion-exchange and reversed-phase separations for the determination of free nucleotide pools in rat tissues and organs. Both modes of retention were necessary to resolve and identify all commonly occurring free major nucleotides. The anion-exchange portion utilized a pH—salt gradient, while the reversed-phase mode required a methanol gradient. Tissue samples were frozen in liquid nitrogen, ground, and extracted with PCA. The extract was neutralized with a freon–amine mixture. The neutralized extract was passed through a ligand-exchange column to remove any nucleosides or bases present. The purified nucleotide fraction was then analyzed by HPLC. Use of such a preliminary sample clean-up can greatly simplify the separation parameters in the nucleotide assay and possibly provide better sensitivity.

A low-temperature extraction method has been reported by Lush et al. [61] for use in the determination of nucleotides in tissues. Use of a TCA—methanol extractant on frozen tissue was found to prevent nucleotide interconversion, which may occur if TCA alone is the extractant. Ion-exchange separation of ATP, ADP, AMP and IMP showed the method to result in 97% recovery. Quantitation was accomplished through the use of radioactively labeled AMP or Ino internal standards. Sample sizes were moderate, being in the 10–100-mg range.

Wynants and VanBelle [62] have developed a method for the simultaneous determination of nucleotides, nucleosides and bases in a wide variety of sample matrices. Samples were lyophilized and extracted with PCA. The extract was neutralized with bicarbonate prior to injection. Use of an organic modifier gradient allowed the reversed-phase separation of 24 compounds to be achieved in 35 min. The sensitivity of the method was approximately 5 ng. The methodology was successfully applied to isochemic heart muscle, skeletal muscle, brain tissue, liver tissue, hepatocytes and erythrocytes. The ability to

simultaneously monitor all three classes of compounds makes the method extremely effective in obtaining an overall view of nucleic acid metabolism in physiological samples.

By use of anion-exchange and reversed-phase ion-pair techniques, Brown et al. [63] have studied the free nucleotide pools of mammalian tissues. Samples were frozen in liquid nitrogen, ground, and extracted with PCA. The extract was neutralized with a freon– amine mixture. The remaining aqueous layer was pre-purified by passage through a copper-loaded ligand-exchange column to remove nucleosides, bases and some amino acids from the nucleotide analytes. The ion-pair method was found to give better separations of cyclic nucleotides. However, the anion-exchange mode provided the best overall results, separating 25 nucleotides in 46 min, although some interferences were observed. The method provided excellent resolution of AMP and NAD, which had been a problem in earlier work [42,48]. Sensitivity was approximately 10 pmol. The

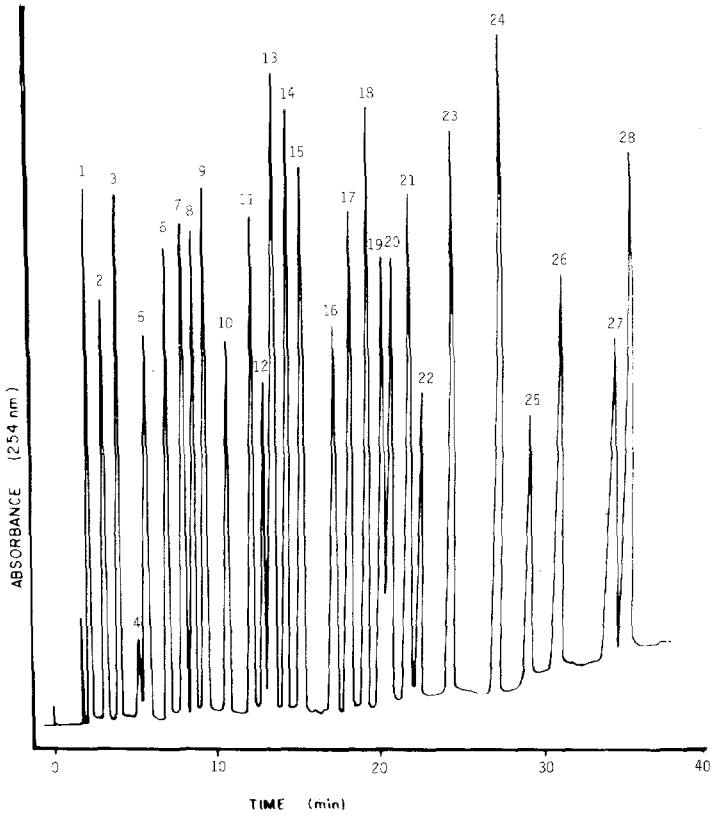


Fig. 4. Separation of 0.1—0.5 nmol of 28 nucleosides, bases, nucleotides, aromatic amino acids and metabolites. Injection volume: 40 μl of a solution $1 \cdot 10^{-5}$ M in each standard. Column: chemically bonded reversed-phase (C_{18}) on 10 μm totally porous silica support. Eluents: low-strength, 0.02 M KH_2PO_4, pH 5.6; high-strength, 60% methanol. Gradient: slope 0.69%/min (0—60% methanol in 87 min), linear. Temperature, ambient; flow-rate, 1.5 ml/min. (Reproduced with permission from ref. 20). Peaks: 1 = Cyt; 2 = Ord; 3 = Ura; 4 = Tyr; 5= Ctd; 6 = Hyp; 7 = Urd; 8= 5-AICAR; 9 = m7Ino; 10 = m7Xao; 11 = m7Guo; 12 = β-NAD; 13 = Ino; 14 = Guo; 15 = Ino; 16 = dThd; 17 = m1Ino; 18 = m1Guo; 19 = m2Guo; 20 = Kyn A; 21 = Ado; 22 = Thb; 23 = m2,2Guo; 24 = Thp; 25 = Dyp; 26 = m6Ado; 27 = 1-3-Prp A; 28 = Caf.

sensitivity might have been slightly improved if the anion-exchange phosphate mobile phase would have been purified to eliminate baseline drift. The method indicated 100% recovery of nucleotides from the ligand-exchange clean-up column. However, decomposition of the nucleotides upon contact with PCA was reported, resulting in an overall recovery of 90%. The paper claimed these losses were due to nucleotide instability in acidic solutions. Perhaps grinding the frozen tissue under liquid nitrogen would have helped to offset some analyte decomposition. Such an approach was not used in the method.

2.3. Physiological fluids

Hartwick et al. [20] reported the use of reversed-phase HPLC for the gradient separation of 86 nucleotides, nucleosides and bases which may appear in human serum. Separation of a standard mixture was completed in approximately 35 min (Fig. 4). A second paper by Hartwick et al. [64] applied the methodology to evaluate the nucleotide, nucleoside and base composition of human serum. Twelve compounds were identified in normal serum, and several

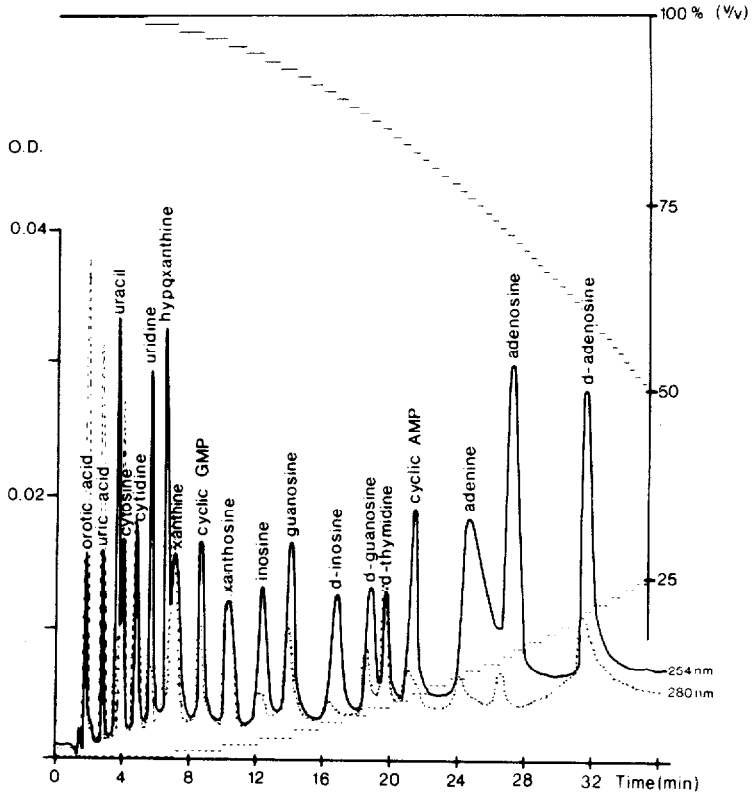


Fig. 5. Elution profile of a test mixture of purine and pyrimidine nucleobases, ribonucleosides, deoxyribonucleosides, cAMP and cGMP. Detection was performed at 254 nm and 280 nm. On the right-hand scale the percentage of each of the solvents used for the elution is indicated. The 0.05 *M* potassium phosphate buffer starts at 100% and ends at 50% (indicated by the step-plot with solid lines), whereas the water and methanol each start at 0%, and end at 25% (indicated by the step-plot with broken lines). (Reproduced with permission from ref. 65.)

variations were observed in serum of patients with various diseases. The samples were subjected to ultrafiltration prior to injection. This preparation method removes proteinaceous material from the serum, but does not dilute the sample or introduce any contaminants. Only free components are contained in the ultrafiltrate, while compounds binding to proteins are not fully recovered.

More recently, a ternary gradient reversed-phase separation of purine and pyrimidine cyclic ribonucleotides, deoxyribonucleosides, ribonucleosides and bases has been reported by De Abreu et al. [65]. The method has been applied to urine, serum and plasma. Nineteen compounds were separated in 35 min. Fig. 5 illustrates the resolution of a standard mixture of these compounds. The serum and plasma samples were treated with PCA prior to injection. Urine samples were simply filtered to remove particulates. Recovery of components was 96%. The method offered a minimum detection limit of 5–10 pmol. The method was used to monitor the urine of a Lesch–Nyhan patient during the course of allopurinol treatment. It was also used for the determination of urinary cAMP in hypoparathyroidism during parathyroid hormone treatment. The advantages of this method include sensitivity, speed and the ability to monitor all classes of compounds in a single chromatographic analysis.

Yoshioka et al. [66] have described an isocratic anion-exchange separation of Ade nucleotides which utilizes fluorometric detection. ATP, ADP, AMP, cAMP and Ado were subjected to pre-column derivatization with bromoacetaldehyde to produce fluorophores. Separation of these analytes was completed in 35 min, provided a minimum detection limit in the femtomole range, and resulted in 100% recovery. The method was applied to human plasma, human urine and rat brain tissue. Advantages of the method are its extreme sensitivity (with easy detection of cAMP in urine), adaptability to various sample matrices, isocratic elution and compatibility with microbore HPLC. The results obtained with the method were comparable to those obtained with radioimmunoassay; however, no radioactive substances are required in the HPLC method.

2.4. Cells

2.4.1. Erythrocytes

Coleman et al. [67] have investigated the Ade deoxy- and ribonucleotide content in erythrocytes of patients suffering from adenosine deaminase (ADA) deficiency. The paper presented three different gradient anion-exchange separations of the nucleotides of interest and was quite confusing, since one of the gradients should have been sufficient to obtain all the results in the study. None of the gradients produced good resolution of ADP from dADP, and ATP from dATP. The results of the study indicated that the total Ade nucleotide content in ADA-deficient erythrocytes was approximately twice that of normal erythrocytes. Also, dATP and dADP were found to be the major abnormal metabolites present in the enzyme-deficient erythrocytes.

Leray [68] has determined purine nucleotides in fish erythrocytes by reversed-phase HPLC. Fish blood was extracted with cold PCA. The extract was neutralized with a freon–amine mixture. The remaining aqueous layer was frozen in liquid nitrogen until the time of analysis. The 12-min isocratic

separation was able to resolve the mono-, di- and triphosphates of Ado and Guo. The resolution between GTP and GDP was minimal. Inspection of the chromatograms also indicated that retention time reproducibility of ATP was poor, ranging from 3.5 min to 5.8 min. Results from this study showed that GTP and ATP accounted for 88 to 99% of total erythrocyte purines in various fish species. The study also grouped 11 fish species into three distinct groups, based upon differences in erythrocyte GTP/ATP ratios.

Schweinsberg and Loo [69] used reversed-phase HPLC to determine ATP, ADP and AMP concentrations in normal human erythrocytes. With a methanol gradient, 17 purines were separated in 35 min. The major components of interest (ATP, ADP and AMP) were essentially baseline resolved. However, GDP and GTP coeluted, as did GMP and IMP. Additionally, Hyp and Gua also coeluted. The study employed the method to calculate concentrations of ATP, ADP and AMP in normal human erythrocytes. Sample preparation consisted of cold PCA extraction, followed by neutralization prior to injection.

Adenine nucleotides in horse erythrocytes were the subject of another investigation by Magnuson and Perryman [70]. The study employed an anion-exchange gradient separation to resolve 14 nucleotides in 55 min. Sample preparation involved a TCA extraction of erythrocytes, instead of extracting whole blood as was done in earlier work [68]. The extract was neutralized with a freon–amine mixture, and the aqueous layer was lyophilized and frozen. Reconstitution with phosphate buffer was performed immediately prior to analysis. CTP was used as an internal standard. Based upon the internal standard, only a 63% recovery of the adenine nucleotides was obtained. Such low recovery can affect sensitivity. The purpose of the study was to use horses as models for altered nucleotide metabolism as found in immune deficiency diseases. Results indicated no significant differences in AMP, ADP and ATP concentrations in four different horse breeds. Also, the nucleotide patterns and concentrations were found to be the same in whole blood and erythrocytes.

Swanson et al. [71] used anion-exchange HPLC for the determination of erythrocyte nucleotides in lead-poisoned rabbits. A pH–salt gradient was used to separate 13 nucleotides in 50 min. Samples were prepared by hemolysis of erythrocytes, rather than extraction. Proteins were precipitated with TCA. The solution was neutralized with a freon–amine mixture and the aqueous layer frozen until the time of analysis (within 5 days). The sample preparation method resulted in very good recoveries, ranging from 85 to 100%. Addition of XMP as an internal standard was used for quantitation of the nucleotides. Results showed drastic increases in erythrocyte UTP, CTP and CDP concentrations as a result of lead poisoning. No significant changes in purine nucleotide concentrations were observed. A decrease in the activity of 5′-nucleotidase was associated with the lead poisoning.

In an effort to improve the resolution obtained by use of pellicular ion-exchange material, Seta et al. [72] employed a styrene–divinylbenzene anion-exchange resin for the separation of UV-absorbing components in human erythrocytes. By use of an acetate gradient, the method easily separated 25 nucleic acid components in 65 min. Plasma was extracted with TCA, which was then injected for the assay. The authors also investigated the use of direct injection of whole blood. This approach was successful; however, the column

required regeneration after every ten injections of whole blood. Even with the disadvantage of frequent column regeneration, direct injection of whole blood may minimize decomposition of free nucleotides.

McKeag et al. [73] have used anion-exchange HPLC for a comparative study of blood nucleotides of various marsupials. A 45-min gradient permitted the separation of a wide variety of compounds. Concentrations of the blood nucleotides of seven marsupials were determined. The chromatographic profiles allowed the classification of the marsupials as either herbivores or carnivores, and it was found that each species studied had a unique nucleotide profile.

The ATP content in bovine erythrocytes was determined by Smith [74]. A gradient anion-exchange separation was used to monitor the ATP levels through the course of various cellular treatments. The separation of AMP, ADP and ATP was completed in 50 min. No information was given concerning the sensitivity or linear detector response range.

Webster and Whaun [75] have studied purine metabolism in parasitic diseases in an effort to develop anti-parasitic chemotherapy. A reversed-phase method using a methanol gradient permitted the separation of ten nucleotides, nucleosides and bases in 35 min. Detection was by both UV absorbance and radioactive monitoring. An alternative anion-exchange separation of eight nucleotides in 45 min was also developed. To minimize baseline drift in the ion-exchange method, the mobile phase phosphate buffer was pre-purified to remove UV-absorbing impurities. The pre-purification consisted of a simple recrystallization step. Human erythrocytes infected with malaria were extracted with PCA. Major nucleotides found in the infected erythrocytes included IMP, ADP and ATP. Resolution of all compounds was acceptable, although examination of the chromatogram suggests an interference may coelute with IMP.

A rapid anion-exchange separation of 13 nucleotides has been reported by Perrett [76]. The system provided adequate resolution of most peaks although NAD and AMP eluted very close to each other and problems may be encountered during quantitation of these two compounds if one is present in a large excess. The paper stated that the method could be used to separate most deoxynucleotides from their respective ribonucleotides. Examples given were of ADA deficiency or purine nucleoside phosphorylase (PNPase) deficiency, where dATP, dADP, dAMP and dGTP accumulate in blood cells. However, the method results in coelution of dGTP and GTP, along with severe peak merging of dADP and ADP. Therefore, modifications are required if quantitative determinations of these compounds are needed.

More recently, Ericson et al. [77] have reported a two-step anion-exchange procedure for the analysis of human erythrocyte nucleotides. The nucleotides separated were AMP, ADP, ATP, GDP, GTP, IMP and NAD. Whole blood was extracted with cold PCA. Two sets of separation conditions were used in the method. One set was used for the 12-min isocratic elution of the nucleoside monophosphates. Another set of isocratic conditions were required for the elution of the nucleoside di- and triphosphates in 21 min. This approach is advantageous only if the monophosphates or the di- and triphosphate mixture are the compounds of interest. However, if separation of all the compounds (i.e. mono-, di- and triphosphates) is desired, this is not the most advantageous

route to follow. A gradient separation would allow resolution of all components in a single run and not require a longer time period than the two separate injections combined. The authors also reported that during the monophosphate assay, the column had to be cleaned after every 40 injections to remove accumulated di- and triphosphates. Although not investigated in the study, use of a guard column, accompanied by frequent changes, would probably eliminate this problem.

Hsu and Chen [78] have reported a rapid gradient anion-exchange separation of ATP, ADP, AMP and Ado in PCA extracts of human blood. The method utilizes a 1-cm column packed with a synthetic resin material. The detection limits are approximately 30 pmol, and the method gave a linear detector response up to approximately 150 nmol. The assay was used to determine the mean concentrations of the analytes in human erythrocytes. Advantages of this system include speed and sensitivity. Results obtained compare favorably with those obtained by other methods used to determine ATP, ADP, AMP and Ado.

An assay for erythrocyte pyrimidine 5′-nucleotidase and deoxypyrimidine 5′-nucleotidase has recently been developed by Cook et al. [79]. In the method enzyme activities were determined by chromatographically monitoring the concentrations of the nucleotide substrates and nucleoside products. The method utilized isocratic reversed-phase separation of the substrates and products. This assay was the first one for the two enzymes which utilized five pyrimidine and five deoxypyrimidine nucleotide substrates.

Another enzyme assay has been developed by Halfpenny and Brown [80] for the simultaneous determination of hypoxanthine–guanine phosphoribosyl transferase (HGPRTase) and PNPase activities in erythrocytes. The isocratic reversed-phase separation of the reaction substrates and products was achieved in less than 5 min. Sample treatment included lysing of the erythrocytes by use of freeze—thaw cycles, followed by incubation with reaction substrates, cofactors and coenzymes. Reaction conditions were chosen to yield zero-order kinetics for both enzymes. Enzyme activities were calculated by measurement of concentrations of the PNPase substrate (Ino) and the HGPRTase product (IMP). The method could have applications in monitoring enzyme activities in enzyme-deficient patients. Advantages of the method include its speed and the ability for the simultaneous determination of two enzyme activities. Current assay methods, such as spectrophotometry, are not capable of such simultaneous activity determinations.

Stocchi et al. [81] have described a reversed-phase separation for the simultaneous determination of adenine and other purine nucleotides in human erythrocytes. Use of a gradient allowed separation of 22 nucleotides and bases in less than 30 min. The resolution was excellent for all compounds. Two methods of sample preparation were discussed. A PCA extraction of erythrocytes was compared to an alkaline extraction coupled with ultrafiltration. The authors found the alkaline extraction—ultrafiltration procedure advantageous because it allowed for the extraction of NADH and NADPH, and was much faster (10 min) than the acid extraction (45 min). Recoveries using the alkaline extraction ranged from 72 to 95%. By a simple adjustment of gradient conditions, it was possible to separate only ATP, ADP, AMP, NAD and NADP in 13 min [82].

2.4.2. Cancer cells

Anion-exchange HPLC has been used by Kinahan et al. to monitor ribonucleoside triphosphates in leukemic cells exposed to methotrexate [83]. The method utilized a phosphate gradient for the separation of 17 compounds in approximately 30 min. However, in an attempt to obtain a more rapid separation, there was severe merging of some peaks, such as UDP, ADP and IDP. Similar merging was also observed for ITP and ATP. The authors stated that up to 50% of the ATP could be lost by three washings of the cells after incubation. Therefore, after cell incubation, the cells were not washed. The medium was simply poured off to eliminate any loss of the nucleotides. The cells were crushed in cold PCA to extract the nucleotides. The supernatant was buffered and frozen until the time of analysis. Results of this study showed a decrease in the cellular nucleoside triphosphate concentrations after exposure to methotrexate. An even more dramatic depletion was observed following treatment with a combination of methotrexate and thymidine.

A more recent publication by Pogolotti and Santi [84] has also described the use of anion-exchange HPLC for the investigation of nucleotides in leukemic cells. Up to 12 nucleotides were separated in 12 min with excellent resolution of all compounds. The method employed ammonium phosphate as the mobile phase buffer. This buffer eliminated baseline drift caused by UV-absorbing impurities often found in potassium phosphate, which is commonly used as a mobile phase buffer. The cells were extracted with TCA, followed by neutralization of the extract with a freon—amine mixture. The aqueous layer containing the nucleotides could be frozen at −70°C for up to three months with no nucleotide decomposition observed. The paper discussed sources of error in quantitation of intracellular nucleotide pools. The error sources included incomplete cell transfer, dilution errors and nucleotide degradation. The authors also noted a short column lifetime, although no specific number of injections was mentioned. Occasional regeneration of the column by use of phosphoric acid and/or EDTA washes could be used to extend the column lifetime.

Garrett and Santi [85] developed an anion-exchange procedure for the determination of deoxyribonucleoside triphosphates in mouse lymphoma cells. Since ribonucleotides were present at much higher concentrations than their deoxy counterparts, removal of the ribonucleotides was necessary to unmask the presence of the deoxyribonucleotides. The removal was accomplished by periodate—methylamine oxidation of the ribonucleotides to form their corresponding bases. This approach resulted in greater than 99.9% removal of ribonucleotides from the PCA cell extracts. The use of methylamine in the oxidation procedure caused the reaction products to elute at the column void volume, thus eliminating possible interferences with the deoxyribonucleotides. Recoveries of the deoxyribonucleoside triphosphates (dCTP, dTTP, dATP and dGTP) ranged from 93% for dGTP to 100% for dATP. The method exhibited flexibility in that it may also be used for the separation of deoxyribonucleoside di- and triphosphates.

Cohen et al. [86] have also studied the ribonucleotide and deoxyribonucleotide content of mouse lymphoma cells. Their method involved an initial separation and collection of the nucleotides, followed by alkaline phosphatase

hydrolysis to the corresponding nucleosides. The nucleosides were then quantified by reversed-phase HPLC. Although this method does allow the measurement of both ribonucleotides and their deoxy counterparts, the procedure is very time consuming, requiring a 3.5-h gradient anion-exchange separation of the nucleotides, followed by a 0.5-h hydrolysis, and a 15-min reversed-phase separation of the resulting nucleosides. The total time is over 4 h per sample, which is unrealistic when dealing with large numbers of samples. However, it does eliminate the problem of ribonucleotides masking the deoxyribonucleotides. Also, the method provided good sensitivity, which was 10 pmol.

A ternary gradient anion-exchange method for the separation, identification and quantitation of nucleotides in lymphocytes and malignant lymphoblasts has been reported by De Abreu et al. [87]. Approximately 95 min were required for the separation of 22 compounds including the mono-, di- and triphosphates of Ado, Ctd, Guo, Ino, Urd and Xao. Good resolution of all the compounds was achieved. Sample preparation consisted of a PCA extraction. Recoveries were 92% or greater. The sensitivity was very good, being 10 pmol for the monophosphates, 20 pmol for the diphosphates and 40 pmol for the triphosphates. High triphosphate/diphosphate ratios indicated the extraction process was reliable and breakdown of the nucleotides was minimized. Results of the study showed that above-normal concentrations of ATP and UTP were present in the malignant cells.

Tanaka et al. [88] have recently reported an improved separation of deoxyribonucleoside triphosphates in ascites tumor cell extracts. The method is essentially that of Garrett and Santi [85], with slight changes made in the mobile phase buffer concentrations. Another modification was made in the order in which reagents were added during the periodate oxidation of the ribonucleotides. The improved method required addition of methylamine after excess periodate was destroyed, in order to eliminate interferences with dTTP or dATP. Sensitivity was good, ranging from 10 pmol for dCTP down to 3 pmol for dATP. Approximately 25 min were required for the separation, with column regeneration necessary after every fifth injection.

2.4.3. Platelets

Reversed-phase ion-pair HPLC has been used by Walseth et al. [89] for the separation of ribonucleoside monophosphates in platelets. In the study many ion-pair reagents were investigated and the authors found the tetrabutylammonium ion to be the best. The separation of six monophosphates (CMP, AMP, GMP, UMP, IMP and XMP) was completed in 30 min. An advantage of this method is for use in the separation and actual isolation of purified ribonucleoside monophosphates. The mobile phase contained only water, methanol and tetrabutylammonium hydroxide. All of these components are volatile and could easily be evaporated from purified fractions.

Another reversed-phase ion-pair separation of platelet nucleotides has been reported by Rao et al. [90]. Although different nucleotides were examined in this study (ATP, ADP, AMP, GTP and GDP), the method is very similar to the one discussed above [89]. Separation was achieved in approximately 25 min with good resolution of all components. Sample preparation consisted of a PCA extraction of the platelets. Recovery of the nucleotides ranged from 94 to

100%. The study also demonstrated the use of radially compressed columns to reduce the separation time to 10 min, instead of 25 min required on a conventional 25-cm column. The quantitative results of this study agree with those obtained by use of a more rigorous, longer gradient separation of platelet nucleotides.

2.4.4. Cardiac cells

Sharps and McCarl [91] have reported on the separation of ribonucleotides in rat heart cells. Requiring less than 75 min for the gradient anion-exchange separation of 12 nucleotides, the method provided baseline resolution of all components in the mixture. The study used a PCA cell extraction, followed by neutralization with a freon—amine mixture. The method was developed to monitor the phosphorylation of adenine nucleotides in cells exposed to halothane anaesthetic.

Purine nucleotides in rat heart cells have also been investigated in another recent study by Burnette et al. [92]. The mono-, di- and triphosphates of Ado and Guo were separated in 30 min by isocratic elution from a 25-cm anion-exchange column. PCA was used to extract the nucleotides from the sample. The method provided a minimum detection limit of 0.25 nmol, and the detector response was linear up to 50 nmol. Advantages of this method are speed and lack of interference from components of enzymatic phosphotransferase reactions. The study showed that the nucleotide content of heart cells rapidly decreased when incubated in a nitrogen atmosphere. The decrease was even more rapid when glucose was absent from the culture medium.

2.4.5. Fibroblasts

Nissinen [93] has developed a gradient anion-exchange procedure for the separation and quantitation of purine and pyrimidine bases, ribonucleosides and ribonucleotides. The method separated 32 compounds in 160 min. Sensitivity was good, with a minimum detection limit of 10 pmol for purines and 40 pmol for pyrimidines. The linear range of the detector response was from 5 to 100 nmol. Borate was used in the mobile phase buffer to complex with sugar phosphates. Ethanol was also a mobile phase additive, used to improve the separation of the ribonucleosides and bases. Unfortunately the method could not resolve GMP and TDP. The same was also true for ATP and XMP. However, the system was able to separate and quantify most of the naturally occurring purine and pyrimidine bases, ribonucleosides and ribonucleotides found in fibroblasts. Use of a packing material composed of large-diameter ion-exchange resin (17 μm) undoubtedly contributed to the extremely long separation and occasional lack of resolution. Use of smaller-diameter packing material would probably have resulted in a faster separation with better resolution.

A more recent method developed by Debetto and Bianchi [94] utilized reversed-phase HPLC to study the purine ribonucleotide pools in fibroblasts. A mixture of 11 compounds (nucleotides, nucleosides and bases) was separated in 22 min by use of an acetonitrile gradient. Baseline resolution of all components was achieved. The linear range of the method was from 5 to 100 pmol, thus providing excellent detection sensitivity. The method was developed to study the effect of hexavalent chromium on purine metabolism. Results indi-

cated that chromium may impair phosphorylation and enzymatic hydrolysis of nucleotides.

2.4.6. Chinese hamster ovary cells

Hunting et al. [95] have used anion-exchange HPLC to separate ribonucleotides in an investigation of the purine and pyrimidine metabolism of Chinese hamster ovary cells. The isocratic separation was performed at 38°C, and allowed for the separation of nucleoside triphosphates and ADP. Recovery was 94% and reproducibility was within 4%. An additional gradient method was also reported for the separation of mixtures of nucleoside mono-, di- and triphosphates.

In contrast, a reversed-phase isocratic separation of 13 nucleotides in Chinese hamster ovary cells has been developed by Taylor et al. [96]. The method was used to separate and quantify the nucleotides in a formic acid extract of the cells. Results of the study yielded ATP/AMP and GTP/GMP ratios found in the cells. Advantages of the technique include the strong retention of cyclic nucleotides and bases. Therefore these compounds do not interfere with the nucleotide assay. Also, use of a formic acid extract resulted in very little breakdown of nucleoside triphosphates into the corresponding mono- and diphosphates. The entire separation was completed in only 16 min, although several peaks were severely merged.

2.4.7. Miscellaneous cells

The separation of ATP, GTP, dATP and dTTP in cultured mouse thymocytes was reported by Donofrio et al. [97]. A phosphate gradient was used in conjunction with a weak anion-exchange column. By observing the concentrations of the individual nucleotides at specific time intervals, the authors were able to monitor nucleotide metabolism and nucleic acid synthesis within the cells.

A separation of adenine nucleotides in liver cell extracts has been developed by Jones [98]. PCA extraction of the cells was followed by a 30-min isocratic reversed-phase separation. Detection was at 340 nm to eliminate detection of interfering chromophores.

A method for separating ribo-, deoxyribo- and cyclic nucleotides in HeLa cells by isocratic reversed-phase HPLC has been published by Martinez-Valdez et al. [99]. Although the ribo and deoxyribo forms of a given nucleotide could be resolved, periodate oxidation for removal of ribonucleotides was required when a general deoxyribonucleotide profile was desired. Compounds separated included the ribo- and deoxyribonucleotides of Ade, Gua, Cyt, Ura and Thy. The method utilized an off-line silica column clean-up of the periodate reaction mixture prior to the deoxyribonucleotide assay. The advantages of the method include isocratic elution and resolution of AMP, TMP and UMP. These compounds have been subject to interferences in a previous method [52].

2.5. Nucleic acid hydrolysates

Wakizaka et al. [100] have used an isocratic reversed-phase technique to determine the deoxyribonucleotide, deoxyribonucleoside and base content of salmon sperm DNA. The DNA was hydrolyzed by formic acid. Chromatograms

show separation of (1) bases Cyt, Gua, Thy and Ade; (2) nucleosides dCtd, dGuo, dAdo and dThd; and (3) nucleotides dCMP, dTMP, dGMP and dAMP. Although a total separation of a mixture of all these components was not shown, the authors claimed to have been able to obtain the composition of 1 μg of DNA.

A more recent study by Christman [101] was involved with the major and modified deoxyribonucleoside monophosphate content of DNA. This study used enzymatic hydrolysis of DNA, rather than an acid hydrolysis. Enzymatic hydrolysis was used to avoid the acid-catalyzed deamination of m5Cyt to Thy. The isocratic reversed-phase separation resolved seven nucleotides in 2 h. The nucleotides separated were dCMP, dUMP, dm5CMP, dTMP, dGMP, dAMP, and dm6AMP. The method was applied to a variety of cells.

Kessler [102] has used reversed-phase HPLC for the separation of nucleoside 2'- and 3'-monophosphates in RNA hydrolysates. The nucleotide isomers are formed during the alkaline hydrolysis of RNA. Use of a reverse ionic strength gradient on a radially compressed column allowed the separation of eight 2'- and 3'-monophosphate isomers and cAMP to be accomplished in 25 min. The method could be applied to a variety of studies investigating RNA composition. The major advantage of this method is the increased speed relative to an anion-exchange method previously used [46].

3. NUCLEOSIDES AND BASES IN VARIOUS MATRICES

Nucleosides and bases are intermediates in the nucleic acid metabolic pathways and are not normally found in significant quantities in cells. Generally, free nucleosides and bases are found in physiological fluids in more abundant concentrations. Due to their similar chromatographic behavior, separations of both nucleosides and bases will be reviewed in this section.

The general approach for nucleoside and base assays involves sample deproteinization or extraction of the components of interest, followed by a reversed-phase separation of the analytes. Since nucleosides and bases usually are more stable than the nucleoside triphosphates, rapid sample decomposition is not a severe problem. Often the major problems include obtaining sufficient sensitivity for the relatively low concentrations of the free nucleosides and bases in biological samples and adequate resolution of the analytes from interfering compounds.

3.1. Standard mixtures

Ehrlich and Ehrlich [103] have described a method for the separation of a standard mixture of the six bases normally found in DNA, along with Ura as well. The separation utilized reversed-phase ion-pair HPLC for the isocratic resolution of the four major bases (Cyt, Ade, Gua and Thy), the two minor modified bases (m5Cyt and m6Ade) and Ura. The method required two sets of conditions to obtain separation of all the components. Thy, m5Cyt, Gua, Cyt and Ura were separated using one set of parameters. By increasing the methanol content of the mobile phase, Ade and m6Ade were separated from the remaining unresolved bases. Such a situation could have easily been avoided

by using a gradient program to resolve all seven compounds in one run. The minimum detection limit for m5Cyt was 10 pmol. No other detection limits or linear ranges were given.

An ion-exclusion separation for the resolution of DNA bases and deoxyribonucleosides has also been reported by Bohacek [104]. The separation of the eight major bases and deoxyribonucleosides required 100 min. An alternate set of parameters was also developed to allow the separation of six major bases and deoxyribonucleosides (all except Cyt and dCtd) in only 10 min. The separation of all eight components resulted in good resolution except for the Cyt—dCtd pair, which overlapped. The rapid method resulted in significant peak merging, especially of Ade and dGuo.

3.2. Tissues

Klabunde et al. [105] have used a reversed-phase technique for the separation of Ado and Ino in rat heart tissue. The gradient procedure allowed separation and quantitation to be completed in 15 min. The method offered a minimum detection limit of 20 pmol of the analytes. Sample preparation involved grinding frozen tissue under liquid nitrogen, followed by an extraction with TCA. The resulting solution was extracted with diethyl ether to remove excess TCA. The neutralized aqueous layer was then acidified and passed through an anion-exchange column to remove any nucleotides present. The purified fraction was then neutralized and frozen until analysis. In spite of the multiple steps in the sample preparation, the procedure was found to yield a 100% recovery of both Ado and Ino. The method was used to investigate use of Ado and Ino as indicators of myocardial hypoxia. Results showed that hypoxia caused a five-fold increase of Ado and a nine-fold increase of Ino concentrations in heart tissues.

Gasser et al. [106] used anion-exchange HPLC to observe the complete degradation of rat plasma Urd in a single pass through the liver. The study showed plasma Urd was replaced from hepatic pools of acid-soluble Urd nucleotides. The anion-exchange procedure was described by Singhal and Cohn in 1972 [107].

In a paper previously reviewed in Section 2.2.3 [54], Morimoto et al. monitored Hyp, Ino and Ado in normal and ischemic gerbil brain tissues. The study showed that concentrations of all three of these compounds increased during brain ischemia. Ado concentrations peaked after 70 min of ischemia, and were 50 to 100 times the normal value. After 70 min, the Ado concentration decreased due to conversion to Ino and Hyp.

3.3. Physiological fluids

3.3.1. Urine

Evans et al. [108] used normal-phase HPLC to monitor urinary pyrimidine nucleosides and bases. The isocratic separation resolved 13 compounds in 20 min. Resolution of Ura and Thd was minimal. The method yielded minimum detection limits ranging from 0.2 ng for Ura to 2.2 ng for Ctd. Detector response was shown to be linear over a concentration range of three orders

of magnitude. Sample preparation consisted of passing the urine through a mixed anion–cation-exchange column to collect neutral and acidic fractions. Recovery using this treatment was reported to range from 92 to 101%. The method was developed to study inborn errors in the urea cycle. By use of this method, normal concentrations of ψ and Ura were determined.

Urinary purines and pyrimidines were the subject of a study, conducted by VanGennip et al. [109], which involved patients with PNPase deficiency. The study utilized a variety of reversed-phase parameters to examine the nucleosides and bases of interest. To make the system compatible with automation, an on-line column switching technique was developed to allow separation of very polar compounds, via a weak anion-exchange column, to be achieved with the same injection used for reversed-phase separation of the non-polar components. As a result of PNPase deficiency, urinary Ino, dIno, Guo and dGuo were found to be elevated while uric acid was below normal levels. The abnormal concentrations were due to the lack of degradation of nucleosides to their bases, caused by the deficiency of PNPase. After treatment with PNPase, the urinary level of uric acid increased while the concentrations of Ino, dIno, Guo and dGuo decreased. The paper is a good example of how HPLC can be automated and used in a clinical study.

Gehrke et al. [110] employed reversed-phase HPLC in a study of urinary modified nucleosides. Results of the study showed elevations of modified nucleosides in various cancers, due to a high turnover of tRNA. The levels of the nucleosides returned to nearly normal values after effective chemotherapy treatment, indicating the therapeutic agent had somehow interrupted the metabolism of the malignant cells.

In a more recent study, Gehrke et al. [111] examined various parameters used in the reversed-phase separation of nucleosides. Factors investigated were mobile phase flow-rate, pH, methanol content and temperature. The authors developed a methanol selectivity factor which allowed prediction of nucleoside retention times using various mobile phase methanol concentrations. Such a factor could prove extremely valuable in the development of nucleoside separation methods. The authors described an isocratic separation of 18 urinary nucleosides, which required 90 min. However, an impurity interfered with ψ, and an alternate set of isocratic conditions was required for the resolution and quantitation of ψ. The problem of multiple separations was then eliminated by use of gradient elution. The gradient program allowed the separation of all 18 nucleosides, including ψ, with no interferences. Quantitation was accomplished by use of 8-bromoguanosine as an internal standard. The paper also reported an isocratic separation of m2,2Guo. Studies showed that urinary m2Guo is degraded by storage of urine at room temperature. The methods were utilized in the analysis of urine samples from patients with various types of cancer.

Anion exchange was used by Seta et al. [112] to separate UV-absorbing constituents in urine. Among the constituents were various nucleosides and bases. Use of small-diameter synthetic resin (5–7 μm) and a linear acetate gradient gave a highly efficient separation of over 100 compounds in 100 min, as shown in Fig. 6. Of the compounds, 33 have been identified. Retention times were highly reproducible, having a relative standard deviation of approximately 1%. Although no actual medical studies were reported in the paper, the

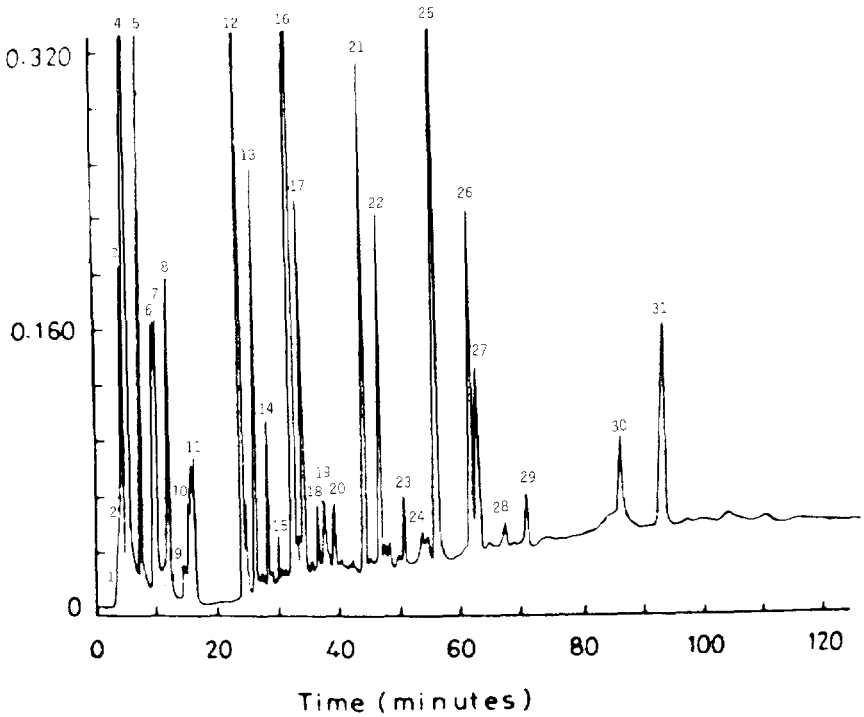


Fig. 6. A 120-min chromatogram of human urine from a normal male subject. Conditions: column, stainless steel (50 cm × 0.4 cm I.D.) packed with Diaion CDR-10; urine sample, 100 μl of a 24-h collection from a 60-kg man; temperature, increasing from 22 to 66°C for the first 25 min, maintained at 60°C until the end of the run; eluent, acetate buffer, pH 4.4, varying in concentration from 0 to 6.0 *M* by linear gradient, average flow-rate, 0.72 ml/min; average pressure, 104 kg/cm^2. (Reproduced with permission from ref. 112.) Peaks: 1 = trigonelline; 3 = creatine, creatinine; 5 = ψ; 7 = pyridoxine; 8 = Ura; 11 = Hyp; 12 = Xan; 14 = urocanic acid; 16 = uric acid; 17 = 2-amino-3-hydroxybenzoylglycine; 19 = dimethyluric acid; 20 = 5-hydroxymethyl-2-furoylglycine; 21 = 2-furoylglycine; 22 = 5-hydroxymethyl-2-furoic acid; 24 = 4-hydroxy-3-methoxyphenylacetic acid; 25 = hippuric acid; 26 = 4-hydroxybenzoylglycine, quinaldic acid; 27 = 3-hydroxybenzoylglycine; 30 = benzoic acid, *p*-hydroxybenzoic acid; 31 = indoleacetic acid; 2, 4, 6, 9, 10, 13, 15, 18, 23, 28, 29 = unknown.

method appears to be an excellent starting point for investigations of urinary nucleic acid components.

Another study by Seta et al. [113] utilized the same system, but employed a perchlorate gradient instead of an acetate gradient. Use of perchlorate gave better sensitivity and less baseline drift. Additionally, acetonitrile or ethanol were added to the mobile phase to aid in the elution of strongly retained components. An additional advantage of this method over the earlier version is the ability to use low-wavelength UV detection to obtain a more general profile.

Schoch et al. [114] have used an isocratic reversed-phase system for the simultaneous determination of 18 unmodified and modified urinary nucleosides and bases. The method utilized a somewhat complicated system of on-line purification and column backflushing. The procedure essentially was divided into separate analyses. By selective retention of bases on one column,

the nucleosides could be separated independently. Then use of a column backflush permitted the bases to be separated. The analysis time was approximately 75 min for each step. The recoveries generally ranged from 90 to 101%, except for m2Gua which was only recovered at 75%. Linear detector responses ranged from 0.4 to 4 nmol. Sensitivity of the method was in the picomole range. Although the method does provide good resolution of the components of interest, it may be somewhat outdated. Use of smaller-particle columns (i.e., 3 or 5 μm) may eliminate the need to use the complicated valve switching technique required to obtain adequate resolution of the analytes.

VanGennip et al. [115] have used a dual-column system for the analysis of urinary purines and pyrimidines. By use of automated column-switching techniques, the authors were able to purify the purine and pyrimidine fractions prior to their reversed-phase or anion-exchange separation. Recoveries in the method ranged from 99 to 106%. The method was used to study levels of urinary purines and pyrimidines in patients suffering from hyperammonemia and defects in pyrimidine catabolism.

Hagemeier et al. [116] reported the synthesis and use of a boronic acid-substituted silica for the separation of ribonucleosides in urine. Although the majority of the paper discussed the production of the silica material, an example of the separation of urinary *cis*-diols (including ribonucleosides) was presented. The column provided good separation of nine identified compounds and many other unidentified compounds in approximately 30 min. The paper demonstrated that such a support can be extremely useful for the group-selective concentration or separation of *cis*-diol compounds.

In a more recent publication by Hagemeier et al. [117], use of an on-line clean-up of urine samples, utilizing column switching, was discussed. The clean-up column was the boronic acid-substituted silica previously used [116]. The column was used to isolate urinary ribonucleosides from other endogenous components. By use of valve switching, these isolated ribonucleosides were then transferred to a reversed-phase column for separation and quantitation. The method resulted in a minimum detection limit of 0.1 nmol, and provided linear detector response ranging from 100 to 2500 pmol. Recovery of the ribonucleosides was above 90%. The advantages of the method are the automation of on-line sample clean-up and speed (25 min). Unfortunately interferences coelute with Ado and m1Guo, making quantitation of these two compounds impossible.

Finally, Giesecke et al. [118] used reversed-phase HPLC to monitor Hyp and Gua in sheep renal output. Although no chromatographic information was provided, the paper does provide an example of how HPLC can be utilized for biochemical studies of nucleic acid components.

3.3.2. Plasma

An isocratic reversed-phase separation of Ino and Ado in human plasma has been reported by Pfadenhauer and Tong [119]. The separation was completed in 14 min and provided good resolution of Ino and Ado from other sample components. Sample treatment included ultrafiltration of the plasma, followed by passage of the ultrafiltrate through a boronic acid gel column to isolate the nucleosides. Nucleosides were eluted from the boronate column with formic

acid, after which the fraction was lyophilized and reconstituted with water prior to injection. The procedure resulted in more than 90% recovery for both Ino and Ado. The precision of the assay was approximately 3% for Ino and 6% for Ado. The method was applied to plasma to monitor drug metabolism.

Isocratic reversed-phase HPLC was also used by Koller et al. [120] to separate Ado and dAdo in human plasma. Requiring less than 10 min, the method gave excellent resolution of the two analytes. The method was linear up to approximately 150 ng of Ado or dAdo. Due to the unstable nature of dAdo in acidic solutions, the plasma was subjected to a barium hydroxide—zinc sulphate extraction process. Such a procedure prevented dAdo from decomposing to Ade and deoxyribose. The resulting solution was injected directly into the chromatograph. The method of sample preparation gave more than 98% recovery of the two analytes. The advantage of this method is the preservation of dAdo during the sample preparation procedure. The assay was applied to plasma of persons suffering from ADA deficiency. The method could be used to observe patient response to ADA replacement treatment by monitoring plasma concentrations of Ado and dAdo.

Wung and Howell [121] developed a chromatographic method for monitoring 5-fluorouracil. However, the separation can also be applied to Xan, Thy and Hyp, in addition to other oxypurines. The isocratic elution required 13 min to separate seven components. Sample treatment was a PCA extraction, followed by neutralization with a freon–amine mixture. Recovery was stated to be 100% for all analytes except allopurinol. Unfortunately Gua coeluted with Hyp under the given conditions. Although the method was primarily developed to monitor the antineoplastic 5-fluorouracil, it could also be useful in monitoring selected plasma nucleosides in cancer patients undergoing 5-fluorouracil treatment.

The determination of plasma and serum Urd levels in mice, rats and humans has been the focus of a recent chromatographic study by Karle et al. [122]. The separation of Urd and an internal standard, m5Ctd, was completed in 13 min. The linear range of Urd was 50 pmol to 5 nmol, with a minimum detection limit of 50 pmol. Two methods of sample preparation were investigated. The first method used TCA precipitation of proteins, followed by removal of excess acid with a freon—amine mixture. The second method used only ultrafiltration of the plasma or serum. The solution resulting from either method was passed through a boric acid column to isolate the nucleosides from any impurities. Both methods of sample preparation produced comparable results. However, due to speed and simplicity, the ultrafiltration technique was the method of choice. The authors found that serum levels of Urd were the same in fasting and non-fasting subjects. These results suggested some autoregulation of serum Urd concentration.

A study investigating the use of Hyp as an indicator of heart hypoxia or ischemia was conducted by Harmsen et al. [123]. Utilizing isocratic reversed-phase HPLC, the authors separated Hyp, Ino, Xan, Ado, Ura, Urd, Xao, Guo and Ade in 20 min. Baseline resolution was achieved for all components, except Urd, Hyp, Xan and Xao. The merging of Xan and Xao was especially severe. The minimum detection limit was 2 pmol, with a linear detector response up to 40 nmol. Sample preparation was accomplished by use of PCA deproteiniza-

tion, followed by neutralization. The resulting solution was then assayed. Recoveries were approximately 75%, which is rather low. Chromatograms of actual sample extracts showed Hyp to be partially merged with two adjacent peaks, which may cause difficulty when quantifying Hyp. However, the authors made no mention of such a problem being encountered.

Zakaria et al. [124] utilized gradient reversed-phase chromatography to investigate the plasma profile of nucleosides, bases and aromatic amino acids found in acute lymphocytic leukemia. The chromatographic parameters produced a 35-min separation of the UV-absorbing constituents in plasma ultrafiltrate. The study showed Urd, Ade, Ino, Gua, Hyp and Xan were all elevated in leukemic samples. Ino was found to be an especially sensitive indicator of the disease severity. The paper provided a good example of how HPLC can routinely be used to monitor markers of disease severity and response to treatment.

Plasma nucleoside and base profiles have been used by Sallis et al. [125] to differentiate various species of marsupials. The chromatographic procedure consisted of a 35-min reversed-phase gradient separation of the analytes. Plasma

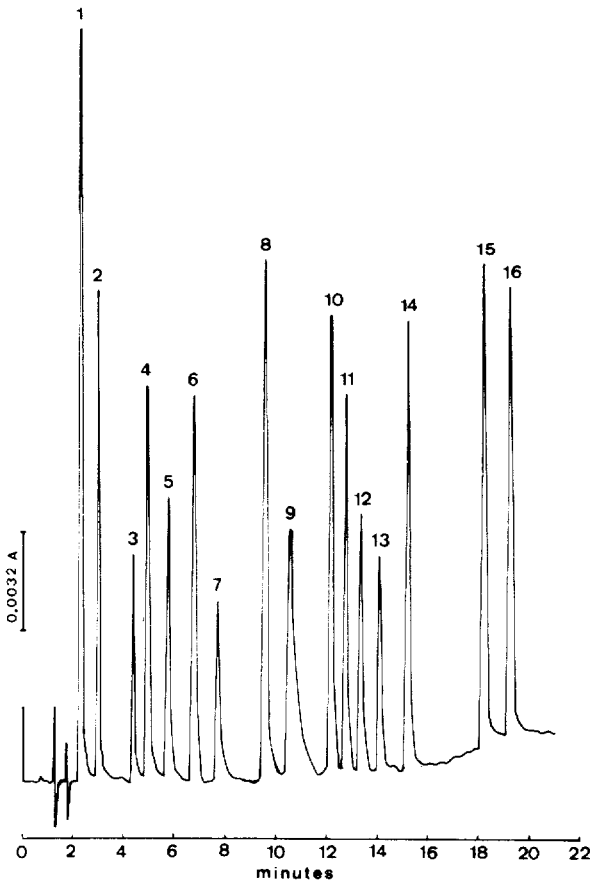


Fig. 7. Chromatographic separation of bases and nucleosides. Peaks; 1 = Cyt; 2 = Ura; 3 = Ctd; 4 = Hyp; 5 = Xan; 6 = Urd; 7 = Thy; 8 = Xao; 9 = Ade; 10 = Ino; 11 = Guo; 12 = dIno; 13 = dGuo; 14 = Thd; 15 = Ado; 16 = dAdo. (Reproduced with permission from ref. 126.)

was deproteinized with TCA, followed by a freon– amine neutralization. The aqueous layer was lyophilized and frozen until the time of analysis. Immediately prior to injection, the samples were reconstituted with water. The study showed that five species of marsupials each had a specific, but reproducible, plasma profile. The purpose of the study was to investigate the metabolism of the Tasmanian Devil, which is of interest due to its high level of plasma acid phosphatase. The system was studied as a possible model for prostatic cancer, which is also characterized by elevated plasma concentrations of acid phosphatase.

Boulieu et al. [126] have monitored purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides in normal plasma and plasma of persons with ADA deficiency. The method consisted of a reversed-phase gradient separation of 16 compounds in under 20 min. A typical separation is shown in Fig. 7. Resolution of all compounds was excellent, and minimum detection limits of 2 to 5 pmol were observed. Sample preparation consisted of a PCA deproteinization, followed by neutralization of the solution. Recoveries of Ade, Ino, dIno, Guo, dGuo, Xao, Ado and dAdo were found to be 97 ± 4%. The high recovery of dAdo is interesting. A previous study [120] found that an alkaline sample treatment was necessary to prevent the decomposition of dAdo, which is unstable in acidic solutions. Although both methods provide excellent recovery of dAdo, the two approaches are in direct conflict. Further study of the stability of dAdo under the different sample preparation conditions is obviously required before any firm conclusions can be reached.

Dwyer and Brown [127] reported on a study investigating the use of electrochemical detection (oxidation mode) for electrochemically active nucleotides, nucleosides and bases. The isocratic reversed-phase elution of eight components was completed in 20 min. Sensitivity for the various compounds ranged from 0.8 pg for GMP to 7.5 pg for XMP. Advantages of the method are the high sensitivity and the selective nature of the electrochemical detector. By adjusting the applied electrode potential, various electroactive analytes may or may not be oxidized. Thus somewhat complex chromatograms may be simplified by proper choice of the applied potential, eliminating peaks which are of no interest.

3.3.3. Serum

The elevation of specific serum nucleosides and bases in breast cancer patients has recently been demonstrated by Krstulovic et al. [128]. The reversed-phase separation afforded excellent resolution of 20 nucleosides and bases in under 30 min. Minimum detection limits ranged from 10 to 20 pmol. The linear detector response range was from 10 pmol to 100 nmol. The serum was deproteinized with TCA, followed by neutralization with Tris base. The resulting solution was frozen until the time of analysis. The advantages of this method are the wide range of linear detector response and the relative speed for the excellent resolution of a large number of compounds. Results of the study showed serum samples from normal and benign breast cyst patients were quite similar. However, breast cancer patients exhibited elevated serum concentrations of Hyp and Guo. Additionally, some of the cancer samples also contained m1Ino and m2Guo.

An enzyme assay for serum acid and alkaline phosphatase, which utilized reversed-phase HPLC, has been developed by Krstulovic et al. [129]. To calculate enzyme activity Ado, which is the product of the enzymatic dephosphorylation of AMP, was quantified. To inhibit 5'-nucleotidase activity, Ni(II) was added to the reaction mixture. Quantitation of Ado was accomplished isocratically in less than 10 min. Advantages of the method include sensitivity at the picomole level, speed and the separation of reaction products from the substrate or other interfering substances.

Assenza and Brown [130] have investigated the use of radially compressed columns for the separation of serum nucleosides and bases. The study showed that under identical gradient conditions, identical separations of a 20-component standard mixture were obtained on both the radially compressed column and a conventional rigid column. The authors state that by increasing the flow-rate, the radially compressed column will provide a separation of equal quality, but in a reduced time. However, a chromatogram obtained with use of increased flow-rate showed loss of resolution for some peaks.

In another study by Assenza and Brown [131] a comparison was made of human and dog serum profiles. The reversed-phase gradient separation was completed in 17 min, and provided good resolution of a number of components in serum. Sample preparation consisted only of ultrafiltration to remove proteinaceous material prior to sample injection. The study showed m5Ctd in the dog serum but not in human samples. Additionally, uric acid was substantially less concentrated in dog samples. There were no significant differences of any other compounds in the two sample populations. The results indicated the major purine metabolic pathway is similar in dogs and humans, even though there are differences in the metabolism of pyrimidines. The data demonstrated that dogs could be used as a model system of human purine metabolism.

Serum and plasma nucleoside and base concentrations in various animals have been studied by Simmonds and Harkness [132], who used isocratic reversed-phase HPLC. One method developed achieved the separation of 16 components in approximately 30 min. Excellent resolution of the compounds was obtained, except for some peak merging of Thy and allopurinol. However, the method was not capable of separating Hyp and Gua. By adjusting the mobile phase pH, Hyp and Gua were resolved in a separate assay which also included Xan, Urd and allopyrinol. As in previous cases, the use of two separate isocratic conditions to obtain analytical data can be more time consuming than use of a single gradient separation. Indeed, the gradient separation of nucleosides and bases reported by Krstulovic et al. [128] provided a separation of most of the compounds involved in the current study. The gradient separation did not require any more time than the isocratic separation of 16 compounds reported in the current method. Thus unless a gradient HPLC system is not available, use of such a gradient (making slight modifications as required) could yield the same information in less time than required for the use of two separate isocratic assays.

Differences in the serum nucleoside and base profiles of smoking and non-smoking beagles have been reported by Assenza and Brown [133]. The study employed a reversed-phase gradient separation to obtain the general profile of

serum ultrafiltrate. The separation was complete in 20 min. Quantitation was performed by use of external standards, although tubercidin was used in certain cases as an internal standard. Recoveries were generally excellent, although no specific information was given in the paper. One exception to the good recovery was tryptophan, at only 12%, due to the characteristic binding of tryptophan to proteins. Analysts should keep in mind that if ultrafiltration is to be used as a means of sample preparation only free concentrations of the analytes are determined, as opposed to total concentration (free plus protein bound) determined with deproteinization using acid. Results of the study indicated several differences between the serum profiles of smoking and non-smoking beagles. Hyp, m5Ctd, Ino and cIMP were all elevated in smoking dogs. Xan was found to be lower than normal in the samples from smoking dogs. These data suggest that exposure to cigarette smoke alters purine metabolism.

Colonna et al. [134] have used HPLC to monitor human serum nucleosides. The separation of five components was achieved isocratically in 20 min. The method was essentially that used by Karle et al. in an earlier work [122]. The procedure included ultrafiltration of serum or use of a boric acid column to clean-up the serum prior to injection. Recovery of ψ was found to be 95% or more. Results from the study indicated a linear detector response range of ψ concentrations from 0.25 up to 2 nmol, with a minimum detection limit of 25 pmol. Data from cancer samples showed that ψ was significantly increased, with the ψ concentration paralleling the progression of the neoplastic disease.

3.3.4. Methods, generally applicable to urine and blood

The papers discussed in this section involve methodologies developed for analyses of urine and blood fluids (i.e., serum or plasma). In contrast, methods discussed in section 3.3.1. were developed specifically for urine, in section 3.3.2. for plasma and in section 3.3.3. for serum.

Putterman et al. [135] have utilized reversed-phase HPLC for the isocratic separation of a variety of urinary and serum nucleotides, nucleosides and bases. Sample preparation consisted of deproteinization with 5-sulfosalicylic acid, followed by buffering. The method provided a linear detector response for up to 150 ng of the various compounds. An unknown compound was found to interfere with Hyp. However, by passage of the sample through a Sephadex G-10 clean-up column prior to injection, the impurity was removed. Recoveries of components were generally over 90%. The procedure was applied to serum and urine samples of cancer patients. Results obtained agreed with those obtained by gas chromatography–mass spectrometry. In some cases the gas chromatographic separations were superior to those obtained by HPLC.

Voelter et al. [136] have applied a reversed-phase ion-pair technique to the separation of selected purines, pyrimidines and their metabolites. A variety of parameters were studied, including counter-ion concentration, mobile phase pH, organic modifier concentration and column temperature. These parameters were optimized to provide separation of eight components of interest, including Hyp, m1Xan and m3Xan. Serum samples were prepared by ultra-filtration. Urine samples were injected directly. The method was designed for use in monitoring gout samples. Comparison of the HPLC results for uric acid compared well with an enzymatic uric acid determination.

Agarwal et al. [137] reported a method to separate a mixture of nine bases, ribonucleosides and deoxyribonucleosides in plasma, urine and cerebrospinal fluid. The method required approximately 25 min for the separation. Resolution of all components was good, with dGuo and dThd overlapping somewhat. Minimum detection limits for Ado and dAdo were 10 pmol each. Ultrafiltration was used for protein removal. Advantages of the technique are speed, resolution and simplicity. No dAdo was detected in normal fluids. However, in samples from a person receiving an ADA inhibitor, dAdo was observed.

The determination of Hyp and Xan concentrations in persons with xanthine oxidase deficiency has been reported by Boulieu et al. [138]. The isocratic reversed-phase separation provided good resolution of Hyp, Xan, m9Xan and allopurinol in less than 10 min. The method was linear for these compounds up to 0.5 nmol. Sensitivity was 5 pmol for Hyp and 10 pmol for Xan. Plasma, to which an internal standard of m9Xan was added, was deproteinized by heating. Urine samples, to which an internal standard was added, were injected without further treatment. The study showed excellent recoveries of the analytes, without losses during sample preparation. The method was simple and rapid. In addition, adequate resolution was obtained between the analytes and endogenous components in the sample matrix.

Miyazaki et al. [139] have utilized reversed-phase HPLC to determine concentrations of urinary and plasma Xan, Hyp, uric acid and various other oxypurines. The separation was isocratic and completed in 15 min. Resolution of all components in a standard mixture was excellent. Resolution of the analytes from components in the sample matrix was also very good. Peak identity was verified by use of gas chromatography–mass spectrometry. No data concerning sensitivity or linear detector response were given. This method was used to monitor the response of patients receiving allopurinol treatment.

Use of a phenylboronate column for the pre-fractionation of ribonucleosides prior to reversed-phase separation has been reported by Hagemeier et al. [140]. The isocratic separation of the purified nucleoside fraction required 30 min. The synthesis of the phenylboronate packing material was described by Hagemeier et al. in an earlier paper [116]. The chromatographic system was automated. Effluent containing the nucleoside fraction was directed from the boronate column to the reversed-phase column by use of a column switching valve. Other components were directed to waste as they exited the boronate column. In addition to automation, another advantage of the system is the high sensitivity, providing a detection limit of 1 pmol/μl. Results concerning the nucleoside content in various physiological fluids were found to be in agreement with an earlier study by Gehrke et al. [111].

The quantitation of plasma and urinary Hyp and Xan in xanthinuria and Lesch–Nyhan patients has recently been completed by Crawhall et al. [141]. Isocratic separation of the analytes was achieved in 11 min. The resolution of Hyp and Xan from endogenous sample matrix components was adequate. Detection was at 208 nm, to minimize the large signal due to uric acid. Although the uric acid signal was decreased, use of low-wavelength UV monitoring resulted in less specific detection of sample components. An interesting technique was used by the authors to obtain the separation. The column was maintained at a sub-ambient temperature to enable the C_8 stationary phase to

accomplish the desired separation. Maintaining sub-ambient column temperatures may be difficult in typical laboratory environments. A more practical approach would have been to operate at ambient temperatures and utilize a C_{18} column. Due to stronger retention characteristics of the C_{18} column, organic modifiers could have been added to the mobile phase to yield the proper retention and selectivity for the assay.

Boulieu et al. [142] have also determined the concentrations of Hyp and Xan in plasma, urine and erythrocytes. The separation was completed in 7 min and provided good peak shape and resolution of uric acid, Hyp, Xan and m9Xan (internal standard). Detection was at 254 nm. Even though a large uric acid response was observed at this wavelength, no interference with Hyp or Xan was observed. Plasma sample preparation consisted of either thermal or TCA deproteinization. Both techniques produced similar results. However, due to simplicity and speed, use of TCA was the method of choice. The study investigated the effect of blood storage temperature on plasma or erythrocyte Hyp and Xan concentrations. Results indicated that the storage temperature of whole blood could produce differences in the concentrations of Hyp and Xan in plasma and erythrocytes.

In a more recent study, Boulieu et al. [143] investigated Hyp and Xan concentrations in the urine, plasma and erythrocytes of persons suffering from xanthinuria. The method used was reported in an earlier paper [138]. The method proved reliable enough that the chromatographic assay could be substituted for diagnostic tissue biopsies.

Although allantoin itself is not a nucleic acid component, it is the final product of purine catabolism in a majority of mammals. Tiemeyer and Giesecke [144] have developed a reversed-phase method for the measurement of allantoin in plasma or urine. The isocratic elution from a 60 cm C_{18} column was completed in 8 min. Recovery of allantoin was 96% in plasma and 98% in urine. No specific biomedical applications were given.

3.3.5. Miscellaneous fluids

Brown et al. [145] have described a method for the determination of uric acid, Hyp and Xan in physiological fluids. The separation utilized reversed-phase ion-pair retention, and was complete in 6 min. The method required PCA deproteinization prior to injection. Advantages of the system are speed and simplicity.

To gain insight into purine metabolism, a variety of physiological fluid nucleosides and bases have been separated by Harkness et al. [146]. Extraction of sample matrices with TCA resulted in a 93% recovery of Hyp.

The determination of UV-absorbing compounds in human saliva was reported by Nakano et al. [147]. The gradient reversed-phase separation required 35 min to resolve numerous compounds in the saliva sample matrix. The study demonstrated the simultaneous presence of biologically important compounds in both serum ultrafiltrate and saliva, although the concentrations in saliva were generally lower. The major advantages of this method are the elimination of sample preparation and the ability to obtain easily and painlessly fluid samples.

Recently, Niklasson [148] has reported a method for the simultaneous

determination of Hyp, Xan, urate and creatinine in cerebrospinal fluid. The isocratic method provided excellent sensitivities, with a minimum detection limit of 3.2 pmol for both Hyp and Xan. Linear detector response was obtained for up to 10 pmol of Hyp and Xan. No sample preparation was required, resulting in recoveries of 103% for Hyp and 99% for Xan. Lack of sample preparation and excellent recoveries make this method very attractive.

3.4. Cells

Halfpenny and Brown [149] have reported an HPLC assay for PNPase in erythrocytes. The method monitors Ino and Hyp as the substrate and product of the enzymatic reaction. The isocratic separation required less than 8 min, and resolved Hyp and Ino from all other components. An example of the separation is shown in Fig. 8. The advantage of such an HPLC enzyme assay is the ability to monitor simultaneously both the forward and reverse reaction products, which cannot be done with spectrophotometric methods. Additionally, the method is fast and inexpensive. No radioactive waste is generated as is in radiochemical assays.

A separation of cellular ribo- and deoxyribonucleosides, especially dThd, has been developed by Harmenberg et al. [150]. The method utilized a cold PCA extraction of cultured African green monkey kidney cells, followed by passage of the extract through a C_{18} solid-phase extraction column to retain the nucleosides. The nucleoside fraction was eluted with methanol and injected for separation of the nucleoside components. The method separated seven compounds in approximately 13 min, with some overlap between Ctd and dCtd. Additionally, tyrosine coeluted with Urd. The minimum detection limit was given at 3 pmol. The method was developed specifically for cells from

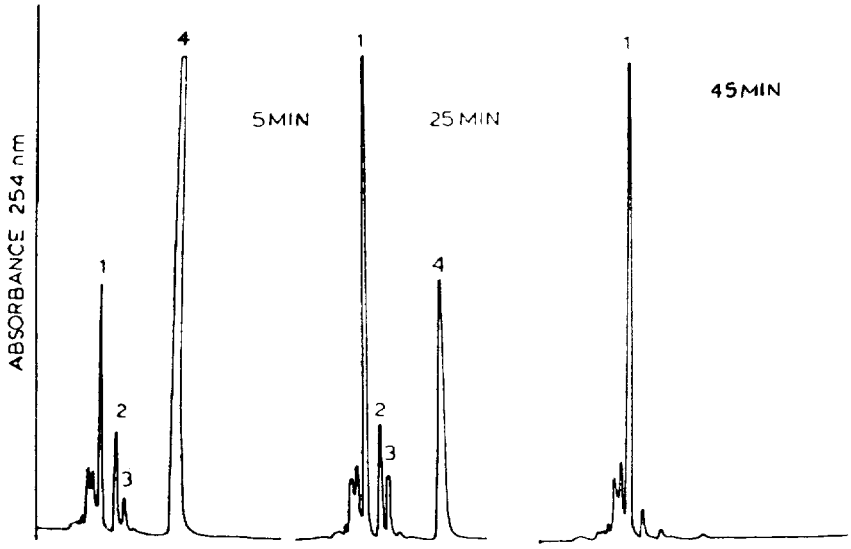


Fig. 8. Reaction of PNPase as a function of time. Chromatograms at time intervals 5, 25 and 45 min show the decrease of the substrate inosine (4) and the increase of the products uric acid (1), hypoxanthine (2) and xanthine (3). (Reproduced with permission from ref. 149.)

tissues and not from cell cultures. Interferences from culture media prevented the method from being applied to cultured cells.

3.5. Nucleic acid hydrolysates

3.5.1. DNA hydrolysates

Kuo et al. [151] utilized reversed-phase HPLC for the determination of major and modified deoxyribonucleosides in DNA. The method used a step gradient for the 72-min separation of six deoxyribonucleosides and four ribonucleosides. The authors found that if RNA contamination was present, two columns in series were required to separate dCtd from Urd and Guo from m5Ctd. However, with no RNA contamination present, one column was sufficient. Also, an isocratic separation was required for the separation of dm6Ado. DNA samples were obtained from calf thymus or salmon sperm. The DNA was enzymatically cleaved to the individual nucleoside components. Approximately 100 μg of DNA were required for the assay. Linear detector responses ranged from 50 to 2500 pmol. The method should be applicable to many types of DNA samples to determine the composition and observe any modifications.

The nucleoside composition of rat brain DNA has also been determined in a study by Heizmann et al. [152]. Gradient elution was utilized in the separation. Use of 2'-deoxy-5-fluorouridine as an internal standard allowed the accurate quantitation of the nucleosides. Samples of DNA were enzymatically cleaved to avoid decomposition of the deoxyribonucleosides.

Kraak et al. [153] used reversed-phase ion-pair HPLC to determine the nucleoside and base composition of calf thymus DNA. The isocratic system employed sodium dodecyl sulfate as the pairing reagent. Only 6 min were required for the separation of 15 compounds. Unfortunately Ura and dThd coeluted under the conditions used. The paper also reviewed the effects of various parameters on the retention characteristics of the nucleosides and bases. The major advantage of the method is the high-speed separation obtained.

The nucleoside composition of formaldehyde-modified DNA has been investigated by Beland et al. [154]. Use of a solvent gradient permitted separation of the hydroxymethyl adducts of the nucleosides. The adducts were formed as a result of exposure of DNA to formaldehyde.

Two recent papers have dealt with the determination of the nucleoside content of rat cerebral DNA. In the first paper, by Hobi et al. [155], DNA was enzymatically digested and the authors utilized a linear gradient for the reversed-phase separation of the resulting nucleosides. Quantitative results in the paper did not agree with results obtained from other methods such as cytofluorometry and UV spectrophotometry. Thus, the paper creates more questions than it answers. However, the authors believed the HPLC technique to be more reliable than the other methods.

The second paper, by Iwasaki et al. [156], does not compare HPLC results with those obtained by other methods. Thus, the uncertainties generated in the previous paper remain. The current HPLC method required 30 min to separate nine compounds. Several disadvantages were inherent in the method. Coelution of Xan with Gua, and dGuo with dThd could cause problems during quantita-

tion. Also, the method did not have minimum detection limits below the levels of nucleosides and bases found in authentic DNA samples. Thus the sensitivity of this method may not be adequate for applications dealing with small quantities of DNA.

Diala et al. [157] have studied DNA base methylation in normal and transformed fibroblasts. The emphasis was placed on m5Cyt. The isocratic method resolved five bases in 14 min. Formic acid hydrolysis of DNA produced the free bases. Resolution of all components was excellent. Results of the study showed no difference in the m5Cyt/Cyt ratio in normal or oncologically modified DNA.

A more recent study, by Diala and Hoffman [158], examined DNA methylation in normal and chemically modified mouse embryo cells. Hydrolysis of DNA samples was carried out in formic acid to prevent decomposition of Cyt or m5Cyt. Isocratic separation of the bases was achieved by the method previously reported [157]. No difference in the percent of methylation was observed between normal and chemically transformed cells. A general disadvantage of the determination of DNA composition by hydrolysis to nucleosides or bases is that no site-specific information regarding methylation can be obtained. Such information could be important in some studies.

The determination of m5Cyt and various other DNA bases has also been the subject of another study, conducted by Citti et al. [159]. Reversed-phase ion-pair HPLC achieved separation of five bases in 20 min. A minimum detection limit of 40 pmol for m5Cyt was obtained. Only 1.3 μg of DNA was required, thus allowing the use of extremely small samples. An advantage of this particular method over a previous ion-pair technique [103] is the ability to resolve all components of interest within a single chromatographic separation.

Eick et al. [160] have also developed a method for the quantitation of m5Cyt in DNA. The reversed-phase ion-pair technique separated six bases in 20 min, which is substantially longer than the time required for the ion-pair separation described earlier by Kraak et al. [153]. Hydrolysis of DNA by formic acid did not cause deamination of Cyt or m5Cyt. Most methods reported have utilized formic acid hydrolysis and not encountered decomposition of Cyt or m5Cyt. However, an earlier paper [101] contends that acid hydrolysis does cause decomposition of m5Cyt and Cyt.

Herron and Shank [161,162] have reported an anion-exchange method for the separation of methylated purines in DNA. The technique was especially developed for monitoring m7Gua and O^6-methylguanine, although Gua and Ade were also resolved. Only 10 min were required to complete the separation. Use of fluorometric detection resulted in minimum detection limits of 7 ng for m7Gua and 150 pg for O^6-methylguanine. The detection limit of O^6-methylguanine was found to be 60 times lower than that possible with UV detection. Use of fluorometric detection is a major advantage of the method. The sensitivity and selectivity were found to be much better than with UV detection. Additionally, the method was flexible enough to be used for assays of m3Ade, m1Ade, m7Ade and m1Gua.

Lawrence et al. [163] have also developed a method for monitoring O^6-methylguanine and m7Gua in DNA. The components were separated in 17 min by use of normal-phase HPLC. Detection was by UV monitoring of the

column effluent. The DNA acid hydrolysate was neutralized and passed through a C_{18} solid phase extraction column for sample clean-up prior to injection. The sensitivity of this method does not appear to be as good as that obtained by Herron and Shank [161,162]. More time was also required for the separation. Also, the minimum detection limit of O^6-methylguanine was not sufficient for actual samples. Thus, fractions containing O^6-methylguanine were collected from multiple injections and reinjected onto a reversed-phase column for quantitation of the O^6-methylguanine. The method was quite tedious and time consuming. As a result, the method of Herron and Shank [161,162] is at present the method of choice.

3.5.2. RNA hydrolysates

The major and modified nucleosides in tRNA have been studied by Davis et al. [164]. The method utilized reversed-phase HPLC. A step gradient was required to separate all components of interest in 60 min. Good resolution of all compounds was achieved, even in the actual sample matrix. Acidic hydrolysis was performed on tRNA from various sources. Following hydrolysis, the solution was passed through a boronate clean-up column to isolate the nucleosides from most other components. The method provided sensitivity in the nanogram range, and was relatively rapid.

The use of HPLC and mass spectrometry has been employed by Agris et al. [165] to study isotopic abundances in tRNA. Following acidic hydrolysis of tRNA, the resulting bases were separated in 24 min on a gradient reversed-

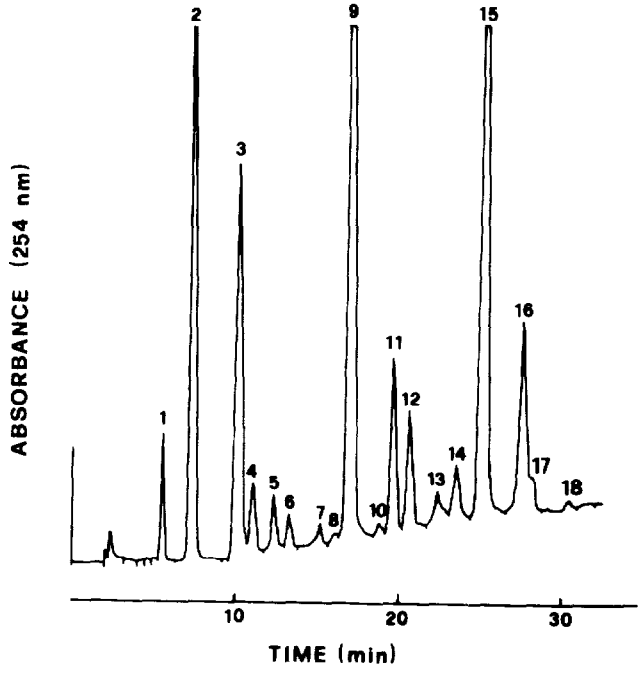


Fig. 9. HPLC profile of nucleosides present in hydrolyzed tRNA. The identified nucleosides are: 1 = ψ; 2 = Ctd; 3 = Urd; 4 = dCtd; 5 = 1-methyladenosine; 6 = m5Ctd; 7 = m7Guo; 9 = Guo; 11 = dGuo; 12 = Thd; 13 = m1Guo; 14 = m2Guo; 15 = Ado; 16 = dAdo; 17 = m2,2Guo; 8, 10, 18 = unknown. (Reproduced with permission from ref. 166.)

phase system. Use of mass spectrometry resulted in the determination of isotopic abundances in the various bases. Such information could be applied to studies of nucleic acid biosynthesis.

The use of HPLC for the determination of ψ in tRNA has been reported by Russo et al. [166]. In addition to ψ, 17 other nucleosides in tRNA hydrolysates were determined, as shown in Fig. 9. Since ψ can be a useful marker for cancer, the method has potential for various studies of neoplastic diseases. Good resolution was obtained for all components except dAdo and m2,2Guo. The minimum detection limit of ψ was 25 pmol, thus enabling small samples of tRNA to be used.

4. SUMMARY

The papers reviewed represent recent progress in HPLC profiling of nucleic acid components in physiological samples. Each method was designed for a particular application and possesses certain inherent advantages and/or disadvantages. Many methods are simply modifications of previous procedures. Although some methodologies appear to be superior to others, there is no "best" method for universal usage. The analyst must use the procedure which is best suited for the particular application at hand. This review is meant to be a starting point for the chromatographer who is comparing and evaluating HPLC methods for a given application.

5. ACKNOWLEDGEMENTS

We thank the Analytical Division of the American Chemical Society and Dr. Robert Barford of the Federation of Analytical Chemistry and Spectroscopy Societies for financial support through an American Chemical Society Analytical Division Summer Fellowship for R.C.S. We also thank the University of Rhode Island Foundation for providing a Graduate Fellowship for R.C.S.

REFERENCES

1 J. Scavennec, Y. Carcassone, J.-A. Gastaut and H.L. Cailla, Cancer Res., 41 (1981) 3222–3227.
2 R.K. Chawla, D.W. Nixon, M. Shoji and D. Rudman, Ann. Int. Med., 91 (1979) 862–864.
3 H.A. Scoble, M. Zakaria, P.R. Brown and H.F. Martin, Comput. Biomed. Res., 16 (1983) 300–309.
4 H.A. Scoble, J.L. Fasching and P.R. Brown, Anal. Chim. Acta, 150 (1983) 171–181.
5 A.H. VanGennip, J. Grift, P.K. DeBree, B.J.M. Zegers, J.W. Stoop and S.K. Wadman, Clin. Chim. Acta, 93 (1979) 419–428.
6 B.S. Mitchell and W.N. Kelley, Ann. Int. Med., 92 (1980) 826–831.
7 A. McBurney and T. Gibson, Clin. Chim. Acta, 12 (1980) 19–28.
8 K.E. Engelman, R.W.E. Watts, J.R. Klinenberg, A. Sjoerdsma and J.E. Seegmiller, Am. J. Med., 37 (1964) 839–861.
9 A.C. Fox, G.E. Reed, H. Meilman and B.B. Silk, Am. J. Cardiol., 43 (1979) 52–58.
10 V. Bianchi, Toxicol., 25 (1982) 13–18.
11 P.R. Brown (Editor), HPLC in Nucleic Acid Research: Methods and Applications, Marcel Dekker, New York, 1984.
12 W.E. Cohn, Science, 109 (1949) 377.

13 M. Uziel, C.K. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77—98.
14 C.A. Burtis, M.N. Munk and F.R. MacDonald, Clin. Chem., 16 (1970) 201—206.
15 Cs. Horváth and S.R. Lipsky, Anal. Biochem., 41 (1969) 1227—1234.
16 J.J. Kirkland, J. Chromatogr. Sci., 9 (1971) 206—214.
17 A. Floridi, C.A. Palmerini and C. Fini, J. Chromatogr., 138 (1977) 203—212.
18 R.P. Singhal and W.E. Cohn, Biochemistry, 12 (1973) 1532—1537.
19 P.R. Brown, S. Bobick and F.L. Hanley, J. Chromatogr., 99 (1974) 587—595.
20 R.A. Hartwick, S.P. Assenza and P.R. Brown, J. Chromatogr., 186 (1979) 647—658.
21 G.E. Davis, R.D. Suits, K.C. Kuo, C.W. Gehrke, T.P. Waalkes and E. Borek, Clin. Chem., 23 (1977) 1427—1435.
22 H.A. Scoble and P.R. Brown, in Cs. Horváth (Editor), High Performance Liquid Chromatography — Advances and Perspectives, Vol. 3, Academic Press, New York, 1983, pp. 1—47.
23 M. Zakaria and P.R. Brown, Anal. Biochem., 120 (1982) 25—37.
24 P.R. Brown and A.M. Krstulovic, Anal. Biochem., 99 (1979) 1—21.
25 P. Brown, R. Hartwick and A. Krstulovic, in G.L. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography II, Marcel Dekker, New York, 1979, pp. 307—335.
26 P.R. Bown, R.A. Hartwick and A.M. Krstulovic, in G.L. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography, Marcel Dekker, New York, 1979, pp. 295—331.
27 K. Lohse, R. Meyer, W. Lin, I. Clark and R. Hartwick, LC Liq. Chromatogr. HPLC Mag., 2 (1984) 266—228.
28 P.R. Brown, A.M. Krstulovic and R.A. Hartwick, Adv. Chromatogr., 18 (1980) 101—138.
29 P.R. Brown, Cancer Invest., 1 (1983) 439—454.
30 P.R. Brown, Cancer Invest., 1 (1983) 527—536.
31 R.W. Zumwalt, K.C.T. Kuo, P.F. Agris, M. Ehrlich and C.W. Gehrke, J. Liq. Chromatogr., 5 (1982) 2041—2060.
32 R.A. Hartwick, D. VanHaverbeke, M. McKeag and P.R. Brown, J. Liq. Chromatogr., 2 (1979) 725—744.
33 M. Zakaria and P.R. Brown, J. Chromatogr., 226 (1981) 267—290.
34 E. Freise, Z. Olempska-Beer and M. Eisenberg, J. Chromatogr., 284 (1984) 125—142.
35 R.T. Pon and K.K. Ogilvie, J. Chromatogr., 205 (1981) 202—205.
36 J.T. Axelson, J.W. Bodley and T.F. Walseth, Anal. Biochem., 116 (1981) 357—360.
37 D.R. Heine, M.B. Denton and T.D. Schlabach, Anal. Chem., 54 (1982) 81—84.
38 K. Yoshida, H. Haraguchi and K. Fuwa, Anal. Chem., 55 (1983) 1009—1012.
39 P. Hubert and J. Porath, J. Chromatogr., 206 (1981) 164—168.
40 J.H. Knox and J. Jurand, J. Chromatogr., 203 (1981) 85—92.
41 M. Glad, S. Ohlson, L. Hansson, M.-O. Månsson and K. Mosbach, J. Chromatogr., 200 (1980) 254—260.
42 E. Juengling and H. Kammermeier, Anal. Biochem., 102 (1980) 358—361.
43 E. Harmsen, P.Ph. de Tombe and J.W. de Jong, J. Chromatogr., 230 (1982) 131—136.
44 O.C. Ingebretsen, A.M. Bakken, L. Segadal and M. Farstad, J. Chromatogr., 242 (1982) 119—126.
45 E.A. Hull-Ryde, R.G. Cummings and J.E. Lowe, J. Chromatogr., 275 (1983) 411—417.
46 E.H. Edelson, J.G. Lawless, C.T. Wehr and S.R. Abbott, J. Chromatogr., 174 (1979) 409—419.
47 J. Maybaum, F.K. Klein and W. Sadee, J. Chromatogr., 188 (1980) 149—158.
48 T.L. Riss, N.L. Zorich, M.D. Williams and A. Richardson, J. Liq. Chromatogr., 3 (1980) 133—158.
49 A.A. Darwish and R.K. Prichard, J. Liq. Chromatogr., 4 (1981) 1511—1524.
50 B. Fleischer, Arch. Biochem. Biophys., 212 (1981) 602—610.
51 P.D. Reiss, P.F. Zuurendonk and R.L. Veech, Anal. Biochem., 140 (1984) 162—171.
52 R.A. Hartwick and P.R. Brown, J. Chromatogr., 112 (1975) 651—662.
53 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chem., 25 (1979) 235—241.
54 K. Morimoto, K. Tagawa, T. Hayakawa, F. Watanabe and H. Mogami, J. Neurochem., 38 (1982) 833—835.

55 T. Yamamoto, H. Shimizu, T. Kata and T. Nagatsu, Anal. Biochem., 142 (1984) 395—399.
56 R.W. Currie, P. Sporns and F.H. Wolfe, J. Food Sci., 47 (1982) 1226—1228.
57 J. Murray and A.B. Thomson, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 209—210.
58 D. Pruneau, E. Wulfert, M. Pascal and C. Baron, Anal. Biochem., 119 (1982) 274—280.
59 E.J. Ritter and L.M. Bruce, Biochem. Med., 21 (1979) 16—21.
60 N.M. Shaw, E.G. Brown and R.P. Newton, Biochem. Soc. Trans., 7 (1979) 1250—1251.
61 C. Lush, Z.H.A. Rahim, D. Perrett and J.R. Griffiths, Anal. Biochem., 93 (1979) 227—232.
62 J. Wynants and H. VanBelle, Anal. Biochem., 144 (1985) 258—266.
63 E.G. Brown, R.P. Newton and N.M. Shaw, Anal. Biochem., 123 (1982) 378—388.
64 R.A. Hartwick, A.M. Krstulovic and P.R. Brown, J. Chromatogr., 186 (1979) 659—676.
65 R.A. de Abreu, J.M. van Baal, C.H.M.M. de Bruyn, J.A.J.M. Bakkeren and E.D.A.M. Schretlen, J. Chromatogr., 229 (1982) 67—75.
66 M. Yoshioka, K. Nishidate, H. Izuka, A. Nakamura, M.M. El-Merzabani, Z. Tamura and T. Miyazaki, J. Chromatogr., 309 (1984) 63—71.
67 M.S. Coleman, J. Donofrio, J.J. Hutton, L. Hahn, A. Daoud, B. Lampkin and J. Dyminski, J. Biol. Chem., 253 (1978) 1619—1626.
68 C. Leray, Comp. Biochem. Physiol., 64B (1979) 77—82.
69 P.D. Schweinsberg and T.L. Loo, J. Chromatogr., 181 (1980) 103—107.
70 N.S. Magnuson and L.E. Perryman, Comp. Biochem. Physiol., 67B (1980) 205—211.
71 M.S. Swanson, C.R. Angle, S.J. Stohs and K.S. Rovang, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 353—361.
72 K. Seta, M. Washitake, T. Anmo, N. Takai and T. Okuyama, J. Liq. Chromatogr., 4 (1981) 129—143.
73 M. McKeag, P.R. Brown and J.D. Sallis, Comp. Biochem. Physiol., 70B (1981) 541—547.
74 R.C. Smith, Comp. Biochem. Physiol., 69B (1981) 505—510.
75 H.K. Webster and J.M. Whaun, J. Chromatogr., 209 (1981) 283—292.
76 D. Perrett, Chromatographia, 16 (1982) 211—213.
77 A. Ericson, F. Niklasson and C.-H. de Verdier, Clin. Chim. Acta, 127 (1983) 47—59.
78 D.-S. Hsu and S.S. Chen, J. Chromatogr., 311 (1984) 396—399.
79 L. Cook, M. Schafer-Mitchell, C. Angle and S. Stohs, J. Chromatogr., 339 (1985) 293—301.
80 A.P. Halfpenny and P.R. Brown, J. Chromatogr., 349 (1985) 275—282.
81 V. Stocchi, L. Cucchiarini, M. Magnani, L. Charantini, P. Palma and G. Crescentini, Anal. Biochem., 146 (1985) 118—124.
82 G. Crescentini and V. Stocchi, J. Chromatogr., 290 (1984) 393—399.
83 J.J. Kinahan, M. Otten and G.B. Grindey, Cancer Res., 39 (1979) 3531—3539.
84 A.L. Pogolotti, Jr. and D.V. Santi, Anal. Biochem., 126 (1982) 335—345.
85 C. Garrett and D.V. Santi, Anal. Biochem., 99 (1979) 268—273.
86 M.B. Cohen, J. Maybaum and W. Sadée, J. Chromatogr., 198 (1980) 435—441.
87 R.A. de Abreu, J.M. van Baal, J.A.J.M. Bakkeren, C.H.M.M. de Bruyn and E.D.A.M. Schretlen, J. Chromatogr., 227 (1982) 45—52.
88 K. Tanaka, A. Yoshioka, S. Tanaka and Y. Wataya, Anal. Biochem., 139 (1984) 35—41.
89 T.F. Walseth, G. Graff, M.C. Moos, Jr. and N.D. Goldberg, Anal. Biochem., 107 (1980) 240—245.
90 G.H.R. Rao, J.D. Peller and J.G. White, J. Chromatogr., 226 (1981) 466—470.
91 E.S. Sharps and R.L. McCarl, Anal. Biochem., 124 (1982) 421—424.
92 B. Burnette, C.R. McFarland and P. Batra, J. Chromatogr., 277 (1983) 137—144.
93 E. Nissinen, Anal. Biochem., 106 (1980) 497—505.
94 P. Debetto and V. Bianchi, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 117—122.
95 D. Hunting, J. Hordern and J.F. Henderson, Can. J. Biochem., 59 (1981) 838—847.
96 M.W. Taylor, H.V. Hershey, R.A. Levine, K. Coy and S. Olivelle, J. Chromatogr., 219 (1981) 133—139.
97 J.C. Donofrio, J. Meier and J.J. Hutton, Cell. Immunol., 42 (1979) 79—89.

98 D.P. Jones, J. Chromatogr., 225 (1981) 446—449.
99 H. Martinez-Valdez, R.M. Kothari, H.V. Hershey and M.W. Taylor, J. Chromatogr., 247 (1982) 307—314.
100 A. Wakizaka, K. Kurosaka and E. Okuhara, J. Chromatogr., 162 (1979) 319—326.
101 J.K. Christman, Anal. Biochem., 119 (1982) 38—48.
102 M.J. Kessler, J. Liq. Chromatogr., 5 (1982) 111—123.
103 M. Ehrlich and K. Ehrlich, J. Chromatogr. Sci., 17 (1979) 531—534.
104 J. Bohacek, Anal. Biochem., 94 (1979) 237—241.
105 R.E. Klabunde, C.L. Winser, C.S. Ito and S.E. Mayer, J. Mol. Cell. Cardiol., 11 (1979) 707—715.
106 T. Gasser, J.D. Moyer and R.E. Handschumacher, Science, 213 (1981) 777—778.
107 R.P. Singhal and W.E. Cohn, Anal. Biochem., 45 (1972) 585—599.
108 J.E. Evans, H. Tieckelmann, E.W. Naylor and R. Guthrie, J. Chromatogr., 163 (1979) 29—36.
109 A.H. VanGennip, J. Grift, S.K. Wadman and P.K. DeBree, in G.L. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography II, Marcel Dekker, New York, 1979, pp. 337—348.
110 C.W. Gehrke, K.C. Kuo, T.P. Waalkes and E. Borek, Cancer Res., 39 (1979) 1150—1153.
111 C.W. Gehrke, K.C. Kuo and R.W. Zumwalt, J. Chromatogr., 188 (1980) 129—147.
112 K. Seta, M. Washitake, T. Anmo, N. Takai and T. Okuyama, J. Chromatogr., 181 (1980) 311—318.
113 K. Seta, M. Washitake, I. Tanaka, N. Takai and T. Okuyama, J. Chromatogr., 221 (1980) 215—225.
114 G. Schoch, J. Thomale, H. Lorenz, H. Suberg and U. Karsten, Clin. Chim. Acta, 108 (1980) 247—257.
115 A.H. VanGennip, E.J. vanBree-Blom, S.K. Wadman, P.K. DeBree, M. Duran and F.A. Beemer, in G.L. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography III, Marcel Dekker, New York, 1981, pp. 285—296.
116 E. Hagemeier, K.-S. Boos, E. Schlimme, K. Lechtenbörger and A. Kettrup, J. Chromatogr., 268 (1983) 291—295.
117 E. Hagemeier, K. Kemper, K.-S. Boos and E. Schlimme, J. Clin. Chem. Clin. Biochem., 22 (1984) 175—184.
118 D. Giesecke, M. Stanassinger and W. Tiemeyer, Can. J. Anim. Sci., 64 (Suppl.) (1984) 144—145.
119 E.H. Pfadenhauer and S.-D. Tong, J. Chromatogr., 162 (1979) 585—590.
120 C.A. Koller, P.L. Stetson, L.D. Nichamin and B.S. Mitchell, Biochem. Med., 24 (1980) 179—184.
121 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704—1708.
122 J.M. Karle, L.W. Anderson, D.D. Dietrick and R.L. Cysyk, Anal. Biochem., 109 (1980) 41—46.
123 E. Harmsen, J.W. de Jong and P.W. Serruys, Clin. Chim. Acta, 115 (1981) 73—84.
124 M. Zakaria, P.R. Brown, M.P. Farnes and B.E. Barker, Clin. Chim. Acta, 126 (1982) 69—80.
125 J.D. Sallis, S.C. Nicol, P. Perrone and P.R. Brown, Comp. Biochem. Physiol., 79B (1984) 391—394.
126 R. Boulieu, C. Bory and C. Gonnet, J. Chromatogr., 339 (1985) 380—387.
127 M.E. Dwyer and P.R. Brown, J. Chromatogr., 345 (1985) 125—133.
128 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chim. Acta, 97 (1979) 159—170.
129 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, J. Chromatogr., 163 (1979) 19—28.
130 S.P. Assenza and P.R. Brown, J. Liq. Chromatogr., 3 (1980) 41—59.
131 S.P. Assenza and P.R. Brown, J. Chromatogr., 181 (1980) 169—176.
132 R.J. Simmonds and R.A. Harkness, J. Chromatogr., 226 (1981) 369—381.
133 S.P. Assenza and P.R. Brown, Anal. Chim. Acta, 123 (1981) 33—40.
134 A. Colonna, T. Russo, F. Esposito, F. Salvatore and F. Cimino, Anal. Biochem., 130 (1983) 19—26.
135 G.J. Putterman, B. Shaikh, M.R. Hallmark, C.G. Sawyer, C.V. Hixson and F. Perini, Anal. Biochem., 98 (1979) 18—26.

136 W. Voelter, K. Zech, P. Arnold and G. Ludwig, J. Chromatogr., 199 (1980) 345—354.
137 R.P. Agarwal, P.P. Major and D.W. Kufe, J. Chromatogr., 231 (1982) 418—424.
138 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, J. Chromatogr., 233 (1982) 131—140.
139 H. Miyazaki, Y. Matsunaga, K. Yoshida, S. Arakawa and M. Hashimoto, J. Chromatogr., 274 (1983) 75—85.
140 E. Hagemeier, K. Kemper, K.-S. Boos and E. Schlimme, J. Chromatogr., 282 (1983) 663—669.
141 J.C. Crawhall, K. Itiaba and S. Katz, Biochem. Med., 30 (1983) 261—270.
142 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, Anal. Biochem., 129 (1983) 398—404.
143 R. Boulieu, C. Bory, P. Baltassat and P. Divry, Clin. Chim. Acta, 142 (1984) 83—89.
144 W. Tiemeyer and D. Giesecke, Anal. Biochem., 123 (1982) 11—13.
145 N.D. Brown, J.A. Kintzios and S.E. Koetitz, J. Chromatogr., 177 (1979) 170—173.
146 R.A. Harkness, R.J. Simmonds, M.C. O'Connor and A.D.B. Webster, Biochem. Soc. Trans., 7 (1979) 1021—1022.
147 K. Nakano, S.P. Assenza and P.R. Brown, J. Chromatogr., 233 (1982) 51—60.
148 F. Niklasson, Clin. Chem., 29 (1983) 1543—1546.
149 A.P. Halfpenny and P.R. Brown, J. Chromatogr., 199 (1980) 275—282.
150 J. Harmenberg, A. Larsson and C.-E. Hagberg, J. Liq. Chromatogr., 6 (1983) 655—666.
151 K.C. Kuo, R.A. McCune, C.W. Gehrke, R. Midgett and M. Ehrlich, Nucleic Acid Res., 8 (1980) 4763—4776.
152 C.W. Heizmann, R. Hobi, G.C. Winkler and C.C. Kuenzle, Exp. Cell Res., 135 (1981) 331—339.
153 J.C. Kraak, C.X. Ahn and J. Fraanje, J. Chromatogr., 209 (1981) 369—376.
154 F.A. Beland, N.F. Fullerton and R.H. Heflich, J. Chromatogr., 308 (1984) 121—131.
155 R. Hobi, M. Studer, F. Ruch and C.C. Kuenzle, Brain Res., 305 (1984) 209—219.
156 S. Iwasaki, H. Tanaka, K. Nakazawa and M. Arima, J. Chromatogr., 341 (1985) 182—186.
157 E.S. Diala, M.M. Plent, D.W. Coalson and R.M. Hoffman, Biochem. Biophys. Res. Commun., 102 (1981) 1379—1384.
158 E.S. Diala and R.M. Hoffman, Biochem. Biophys. Res. Commun., 104 (1982) 1489—1494.
159 L. Citti, P.G. Gervasi, G. Turchi, L. Mariani and M. Durante, J. Chromatogr., 261 (1983) 315—319.
160 D. Eick, H.-J. Fritz and W. Doerfler, Anal. Biochem., 135 (1983) 165—171.
161 D.C. Herron and R.C. Shank, Anal. Biochem., 100 (1979) 58—63.
162 D.C. Herron and R.C. Shank, Cancer Res., 40 (1980) 3116—3117.
163 J.F. Lawrence, R. Leduc and J.J. Ryan, Anal. Biochem., 116 (1981) 433—438.
164 G.E. Davis, C.W. Gehrke, K.C. Kuo and P.F. Agris, J. Chromatogr., 173 (1979) 281—298.
165 P.F. Agris, J.G. Tompson, C.W. Gehrke, K.C. Kuo and R.H. Rice, J. Chromatogr., 194 (1980) 205—212.
166 T. Russo, F. Salvatore and F. Cimino, J. Chromatogr., 296 (1984) 387—393.