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REVIEW

HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHY OF NUCLEOTIDES, NUCLEOSIDES AND BASES

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CONTENTS

1. INTRODUCTION

Nucleotides, nucleosides and bases are essential constituents of nucleic acids and enzyme cofactors required for the proper functioning of cells, tissues and organs. The importance of nucleotides, nucleosides and bases is demonstrated by the severe symptoms which result from defects in purine or pyrimidine metabolism, such as mental retardation, cardiovascular diseases, renal failure, gout and toxemia [1].

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The development of high-performance liquid chromatography (HPLC) has facilitated the isolation and quantification of the nucleic acid constituents in biological fluids and tissues; separations which previously required several hours by open-column methods can be achieved rapidly using HPLC. With the on-line detection systems, the characterization as well as the accurate quantification of the solutes of interest can be accomplished.

The three major HPLC modes used in the analysis of the nucleotides, nucleosides and bases are: ion-exchange, either cation or anion; reversed-phase and ion-pairing. In cation exchange, the stationary phase contains fixed anionic sites. These interact electrostatically with cationic solutes which are thus retained. Conversely, in anion exchange, the fixed sites are positively charged and anions are retained [2]. In the reversed-phase mode which is used for the separation of non-polar and slightly polar compounds, hydrophobic interactions determine the extent of retention [3, 4]. The more polar or ionic solutes, which favor the aqueous eluent, elute faster in reversed-phase HPLC. In a technique known as ion-pairing chromatography, compounds are added to the mobile phase which contain both a lipophilic moiety that can interact with the non-polar reversed-phase stationary phase and an ionic moiety that can pair with ionic compounds of an opposite charge. Thus, greater retention of charged solutes can be achieved on reversed-phase systems [5].

Nucleosides as bases are relatively weak bases (low pK_{ab} values) and weak acids (high pK_{aa} values) (Table 1) [6]. Since these compounds are positively

TABLE 1

pK_{ab} AND pK_{aa} VALUES OF THE BASES AND NUCLEOSIDES

Data taken from ref. 6. Values are mentioned only for the first gain (pK_{ab}) or loss (pK_{aa}) of a proton.

	pK_{ab}	pK_{aa}
Bases		
Adenine (Ade)	4.15	9.8
Guanine (Gua)	3.2	9.6
Hypoxanthine (Hyp)	2.0	8.9
Xanthine (Xan)	0.8	7.5
Cytosine (Cyt)	4.45	12.2
Uracil (Ura)	−3.4*	9.5
Thymine (Thy)	— *	9.9
Nucleosides		
Adenosine (Ado)	3.5	12.5
Guanosine (Guo)	1.6	9.2
Inosine (Ino)	1.2	8.8
Xanthosine (Xao)	<2.5	5.7
Cytidine (Cyd)	4.15	12.5
Uridine (Urd)	— *	9.2
Thymidine (Thd)	— *	9.8

*Extremely low pH needed for the protonation of the species. Since both nitrogens in uracil and thymine are involved in amide tautomerism, very little basic strength remains (basic N not involved in tautomerism).

charged below their pK_{ab}, they can be separated by cation exchange at these pH values. Since they are neutral between the pK_{ab} and pK_{aa}, they can be analyzed by reversed phase. Above their pK_{aa}, they are negatively charged so they can readily be chromatographed on anion-exchange columns.

On the other hand, the nucleotides are strong acids. At a pH of 2.0, the monophosphates have one negative charge on their phosphate moiety, the diphosphates two, and the triphosphates three. At a pH of 7.0 and above, the nucleotides gain an additional negative charge due to the secondary phosphate dissociation. Thus, these compounds naturally lend themselves to separations on anion exchangers. Recently, attempts have been made to separate the nucleotides by ion-pair reversed-phase chromatography due to the adaptability of these stationary phases to rapid solvent changes.

We will briefly review the steps required for the determination of nucleotide, nucleoside and base levels in biological samples, present some of the chromatographic separations achieved with each of the different modes, and illustrate applications of these separations in the clinico-biochemical field.

2. ANALYSIS OF NUCLEOTIDES, NUCLEOSIDES AND BASES

A. Sample preparation

a. Extraction from cells. In order to study the free nucleotide, nucleoside or base content of a certain volume or number of cells, it is important to extract those compounds into a liquid medium. Desirable reagents for extraction procedures are those that can: (1) lyse the cell; (2) precipitate the protein (to stop the enzymatic degradation of the nucleotides as well as prevent the clogging of the chromatographic column); (3) give the best recovery of the compounds of interest; and (4) provide a neutral environment for the storage of those compounds.

Perchloric acid is commonly used to extract the nucleotides from biological cells [7—13]. Normally, the resulting acidic supernatant is neutralized with potassium hydroxide [8—12] or an amine—Freon^{R} solution [13, 14]. When only the deoxyribonucleotides are of interest, the neutralized extract can be treated with periodate—methylamine. This will ensure the removal of the ribonucleotides from the sample [9].

b. Extraction from biological fluids. Protein removal is probably the most important step in the analysis of nucleosides and bases in biological matrices. Conventionally, perchloric acid or trichloroacetic acid have been used. However, recently, serum [15—17], plasma [18] and urine [15, 16] samples have been ultrafiltered through membrane cones which can retain high-molecular-weight proteins. This method is preferred since it does not alter the pH of the medium, dilute the sample or interfere with the UV absorbance of sample constituents.

Gehrke et al. [19] developed a novel extraction procedure for the analysis of ribonucleosides in urine. The samples are passed through a boronate gel column. The ribonucleosides are retained on the column as *cis*-diol boronate complexes and subsequently eluted with 0.1 *M* formic acid.

B. Chromatography

Today, most chromatographic systems comprise a solvent delivery system, a gradient programmer, an injector, a column, several detector devices, one or more recorders and an integrator.

Within a chromatographic mode, the optimization of a given separation usually requires modification of the mobile phase rather than the stationary phase. The pH of the eluent plays a major role in determining the extent of dissociation of a solute [20, 21]. Charged compounds have classically been resolved by ion exchange. The larger the negative charge on a solute, the greater the retention on an anion exchanger. Elution of highly charged compounds requires eluents of high ionic strength, hence the use of ionic strength gradients in nucleotide separations.

On the other hand, non-polar compounds are retained on hydrophobic reversed-phase stationary phases. Thus, the nucleosides and bases, predominantly neutral between their pK_{ab} and pH 7 (limit of the stationary phase), lend themselves to separations by reversed-phase liquid chromatography. Gradients are often used to decrease the polarity of the system and speed up the elution of those compounds which are retained the longest.

In the ion-pair mode used for the separation of nucleotides on reversed-phase columns, the pH of the mobile phase, percentage organic modifier, and especially the nature and concentration of the pairing agent need to be considered to achieve the proper resolution of the solutes of interest [22].

C. Characterization of chromatographic eluates

This step is extremely important in the HPLC analysis of biological mixtures. Initially, compounds are identified by retention times and co-chromatography with the reference compounds. In addition, absorbance ratios at various wavelengths [23], stopped-flow UV spectra [24] or fluorimetric selectivity [25, 26] may be determined. A scintillation flow-monitor may be used for the detection of radioactive compounds [11]. Chemical tests, such as sample treatment with periodate, are used to identify *cis*-diolic compounds such as ribonucleosides and ribonucleotides [25]. Specific enzymatic tests [27, 28] can also be performed (Table 2) and the decrease in the peak area of the substrate and/or increase in the area of the product can be determined.

In the future, when the problems of interfacing liquid chromatography with mass spectrometry are resolved, the tandem operation of these two techniques will become one of the most powerful tools for the combined separation and identification of eluates.

D. Quantification

After the separation and identification of chromatographic eluates, their quantification can be achieved. One must first determine the response factor R (moles/area) of the reference compounds. This is essentially the slope of the calibration curve, in which known amounts of a compound, injected from solutions of different concentrations, are plotted against the peak area obtained. Using the external calibration method, one can write

$$\text{Amount of X in sample} = (\text{area})_X \cdot R_{ES} \cdot \text{dilution factor} \quad (1)$$

TABLE 2

EXAMPLES OF ENZYMATIC TESTS PERFORMED IN THE IDENTIFICATION OF NUCLEOTIDES, NUCLEOSIDES AND BASES

Enzyme	Substrate	Product
Assays for specific group of substrates		
5'-Nucleotidase	Nucleoside monophosphate	Nucleoside
Alkaline and acid phosphatase	Nucleotide	Nucleoside
Purine nucleoside phosphorylase	Nucleoside + P_i	Base
Assays for specific substrate(s)		
Xanthine oxidase	(1) Hypoxanthine (2) Xanthine	Xanthine Uric acid
Adenosine deaminase	Adenosine	Inosine
Adenosine kinase	Adenosine	Adenosine monophosphate
Guanase	Guanine	Xanthine

where X and ES refer to the unknown and external standard, respectively.

The internal calibration method is often more desirable when variable recovery of compounds from biological matrices is involved. A known amount of internal standard is added to the sample prior to the extraction step. Thus

$$\text{Amount of X in sample} = \frac{(\text{area})_X \cdot R_X}{(\text{area})_{IS} \cdot R_{IS}} \cdot (\text{amount})_{IS} \cdot \text{dilution factor} \qquad (2)$$

where X and IS refer to the unknown and internal standard, respectively.

The major requirement for this method is that all compounds quantified have the same recovery as the internal standard itself — a condition often hard to achieve.

3. SELECTED CHROMATOGRAPHIC SEPARATIONS

A. Nucleotides

a. Ion exchange. The pioneering studies of Cohn [29], who separated nucleic acid components by ion-exchange chromatography, of Anderson [30], who developed an automated effluent monitoring device, and of Kirkland and Felton [31, 32] and Horváth and co-workers [33, 34], who investigated the parameters which affect column efficiency, marked the beginning of a new era in the science of chromatography. The introduction, in the late sixties, of stainless-steel columns packed with pellicular material (ion-exchange resins coated on 50-μm glass spheres) capable of withstanding pressures of up to 27 MPa (4000 p.s.i.), combined with the use of pumps to force the mobile phase through the chromatographic column, made possible the rapid separation of complex mixtures [33].

The ion-exchange mode of HPLC gained considerable importance for the

separation of nucleotides in investigations of the composition and structure of the nucleic acids. Since the nucleotides also regulate various cellular functions, the quantification of the free nucleotide concentrations in cells was essential in biomedical research. The early work of Horváth et al. [33], who achieved the separation of mono-, di- and triphosphate ribonucleotides in 90 min on pellicular anion-exchange resins illustrated the power and great potential of the new HPLC technique. Prior to that, the same separation on conventional open columns required a minimum of 20 h [33]. During this long period of time, several compounds could decompose.

The development of microparticulate packings, in 1973, set another milestone in the history of chromatography. The efficiency of the column was increased while sufficient sample loading capacity was maintained. Hartwick and Brown [35] achieved excellent resolution of the mono-, di- and triphosphate 5'-nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine, using a microparticulate, chemically bonded strong anion exchanger (Partisil 10-SAX, 10 μm). The low-strength eluent consisted of 0.007 *F* KH_2PO_4 (pH 4.0) and the high-strength eluent of 0.25 *F* KH_2PO_4, 0.25 *F* KCl (pH 4.5). The conditions of analysis were: a 15-min elution with the low-concentration buffer followed by a 45-min linear gradient to 100% of the high-strength buffer at a flow-rate of 1.5 ml/min (Fig. 1).

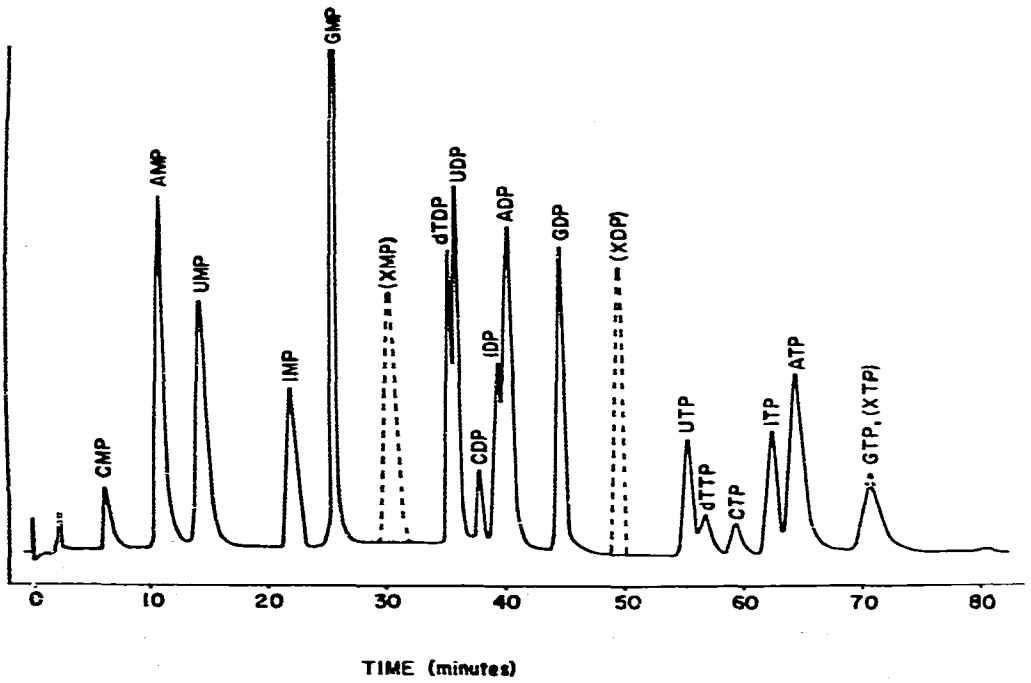

Fig. 1. Separation of mono-, di- and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine. Column, Partisil 10-SAX; temperature, ambient; detector sensitivity, 0.08 a.u.f.s. Eluents: (low 0.007 *F* KH_2PO_4, pH 4.0; (high) 0.25 *F* KH_2PO_4, 0.50 *F* KCl, pH 4.5. Gradient, linear, 0—100% of high-concentration eluent in 45 min; flow-rate, 1.5 ml/min. Dashed lines indicate elution positions of XMP, XDP and XTP. (Ref. 35.)

More recently, the 2′-, 3′-, 5′-ribonucleotides and cyclic ribonucleoside monophosphates of cytosine, adenine and guanine [7], the ribo- and deoxyribonucleoside triphosphates [8], as well as mono- and diphosphates [13] have been resolved using ion exchange. Floridi et al. [12] succeeded in analyzing simultaneously a mixture of bases, ribonucleosides and ribonucleotide mono-, di- and triphosphates. Similar separations were later achieved by Bakay et al. [11] and Nissinen [36]. The chromatographic conditions for these analyses are listed in Table 3.

b. Reversed phase. The use of the ion-exchange mode for the separation of the nucleotides had several disadvantages. Most analyses required one or two hours. The use of buffer gradients resulted in considerable baseline drift. An appreciable amount of time was needed to equilibrate the column at initial conditions after a chromatographic run. The separations were not always reproducible [37]. On the other hand, the development by Halász and Sebastian [38] of chemically bonded, microparticulate reversed-phase packing materials resulted in enhanced stability of stationary phases. In addition, equilibration times were very short and the chromatograms obtained extremely reproducible [28].

Since the nucleotides are negatively charged, their retention on reversed-phase systems seemed, a priori, impossible. However, several researchers attempted to separate, if not all compounds at once, a selected group of nucleotides with similar characteristics.

Using a low pH buffer, Horváth et al. [21] resolved some ribonucleoside monophosphates on a Partisil 10/25 ODS-2 column (Whatman). Krstulović et al. [39] separated the cyclic nucleoside monophosphates on a μBondapak C_{18} column (Fig. 2). Finally, Schweinsberg and Loo [40] were able to resolve ATP, ADP and AMP from other nucleotides, nucleosides and bases. Since adenine nucleotides predominate in erythrocytes, the method can be applied to the study of their levels in lysates with no interference from other red blood cell constituents. Chromatographic conditions for these analyses are given in Table 4.

c. Ion-pairing. The reversed-phase mode had obvious limitations. Although rapid separations of certain types of compounds were achieved, all ribonucleoside mono-, di- and triphosphates could not be resolved simultaneously as many of these compounds were not retained on the column. The addition to the mobile phase of molecules with a hydrophobic moiety that could adsorb on to the stationary phase and an ionic group that could pair with the negatively charged nucleotide, resulted in the greater retention of nucleotides on reversed-phase columns. This chromatographic technique was termed ion-pairing.

In 1977, Hoffman and Liao [41] succeeded in separating the ribonucleoside mono-, di- and triphosphates of cytosine, uracil, guanine and adenine in 35 min on Spherisorb ODS (10 μm, Spectra-Physics; re-coated to 13.9% by weight carbon loading). Gradient elution was used and the mobile phase contained tetra-*n*-butylammonium hydrogen sulfate as the ion-pairing reagent.

Recently, shorter separations for these compounds have been achieved by Juengling and Kammermeier [37], and by Knox and Jurand [22], who used eluents with higher pH values and greater initial amounts of the organic modifier.

TABLE 3

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING ION EXCHANGE

Compounds* resolved	Stationary phase	Mobile phase	Temp. (°C)	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
2'-, 3'-, 5'-nucleotides and cyclic ribonucleotides of Cyt, Ade and Gua	Micropak AX-10 (30 cm × 4 mm, 10 μm, Varian)	0.01 *M* KH_2PO_4 (pH 3.0)	Ambient	2.0	40	7
Ribo-monophosphates and deoxyribonucleoside triphosphates of Cyt, Ura, Ade and Gua	Two Partisil 10-SAX in series (25 cm × 4.6 mm, Whatman)	A: 0.2 *M* $NH_4H_2PO_4$ (pH 3.2) B: 0.6 *M* $NH_4H_2PO_4$ (pH 4.4) Linear 2-h gradient from A to B	40	1.0	105	8
Ribo- and deoxyribonucleoside mono-, di- and triphosphates	Aminex A-29 (30 cm × 4 mm, Bio-Rad)	A: 0.025 *M* sodium citrate (pH 8.2) B: 0.5 *M* sodium citrate (pH 8.2) 30 min A followed by 2-h linear gradient to B	50	0.3	220	13
Bases, nucleosides, nucleotides — mono-, di- and triphosphates	Aminex A-14 (20 ± 3 μm, filled in 50 × 0.6 cm thick-walled glass column, Bio-Rad)	A: 0.1 *M* 2-methyl-2-amino-1-propanol (MAP), 0.1 *M* NaCl (pH 9.9) B: 0.1 *M* MAP, 0.4 *M* NaCl (pH 10) Linear gradient by placing 200 ml of A in mixing chamber and 200 ml of B in reservoir	55	100 (ml/h)	225	12
Bases, nucleosides, nucleotides	Aminex A-25 (17.5 ± 2 μm, in a 50 × 0.2 cm column, Bio-Rad)	A: 0.08 *M* $Na_2B_4O_7$, 0.05 *M* NH_4Cl (pH 9.1) B: 0.01 *M* $Na_2B_4O_7$, 0.5 *M* NH_4Cl (pH 9.0) 45 ml of A in cylindrical mixing chamber and 45 ml of B in reservoir	60	0.5	125	11

*For abbreviations, see Table 1.

TABLE 4

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING REVERSED PHASE

Compounds* resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Cyt, Ura, Ade and Gua ribonucleoside monophosphates	Partisil 10/25 ODS-2 (Whatman)	Phosphate buffer (pH 2.2)	25°C	4.0	3	21
Cyclic nucleoside monophosphates of Cyt, Ura, Gua, Hyp and Ade	μBondapak C_{18} (Waters)	A: 0.02 *M* KH_2PO_4 (pH 3.7) B: methanol—water (3 : 2) Gradient linear from 0% to 25% B in 30 min	Ambient	1.5	25	39
ATP, ADP, AMP and other nucleotides, nucleosides and bases	μBondapak C_{18}	A: 0.06 *M* K_2HPO_4, 0.04 *M* KH_2PO_4 (pH 6.0) B: 3 × [0.08 *M* K_2HPO_4, 0.05 *M* KH_2PO_4 (pH 6.0)], 1 × methanol Gradient concave from A to B in 30 min	Ambient	1	35	40

*For abbreviations, see Table 1.

TABLE 5

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING ION-PAIRING

Compounds* resolved	Stationary phase	Mobile phase**	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Ribonucleoside mono-, di- and triphosphates of Cyt, Ura, Gua and Ade	Spherisorb *ODS* (Spectra-Physics) recoated	A: 0.025 *M* TBHS, 0.05 *M* KH_2PO_4, 0.08 *M* NH_4Cl (pH 3.9) B: 0.025 *M* TBHS, 0.1 *M* KH_2PO_4, 0.2 *M* NH_4Cl (pH 3.4) + 30% methanol 40-min concave gradient	–	1	35	41
Ribonucleoside mono-, di- and triphosphates of Ura, Gua and Ade	LiChrosorb RP-8 (Merck)	Acetonitrile—water (24:86), 0.65% KH_2PO_4, 0.3% TBAP (pH 5.8) Isocratic	–	2	8	37
Ribonucleoside mono-, di- and triphosphates of Gua and Ade	ODS-Hypersil (Shandon Southern Products)	Methanol—water (12:88), 75 m*M* phosphate, 1.25 m*M* C11AA (pH 5.65) Isocratic	25°C	–	15	22
Ribonucleoside mono-, di- and triphosphates of Ura, Gua and Ade	$Co(en_3)^{3+}$ bonded to silica	0.037 *M* $Na_2HPO_4 \cdot 7\,H_2O$ with 1 m*M* $MgSO_4 \cdot 7\,H_2O$ (pH 6.4)	–	1	15	42

*For abbreviations, see Table 1.

**TBHS = tetra-*n*-butylammonium hydrogen sulfate; TBAP = tetrabutylammonium phosphate; C11AA = aminoundecanoic acid.

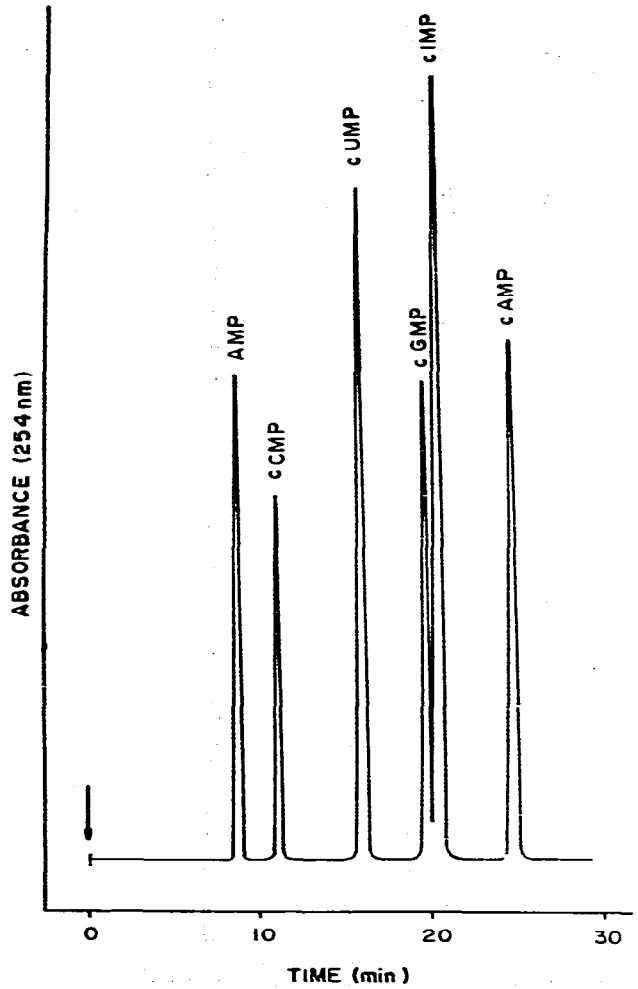

Fig. 2. Separation of reference compounds detected at 254 nm. AMP, adenosine 5'-monophosphate; cCMP, cytidine 3', 5'-cyclic phosphate; cUMP, uridine 3',5'-cyclic phosphate; cGMP, guanosine 3',5'-cyclic phosphate; cIMP, inosine 3',5'-cyclic phosphate. Concentration, approx. 10 nmol each. Chromatographic conditions: column, μBondapak C_{18}; low-concentration eluent, 20 mmol/l KH_2PO_4 (pH 3.7); high-concentration eluent, methanol—water (3:2, v/v); gradient, linear from 0% to 25% of the high-concentration eluent in 30 min; flow-rate, 1.5 ml/min; temperature, ambient; attenuation, 0.1 A full-scale. (Ref. 39.)

Whereas these separations were achieved on reversed-phase columns with C_{18} or C_8 chains bonded to the silica backbone, Chow and Grushka [42] resolved a nucleotide mixture on a column in which a $Co(en_3)^{3+}$ moiety had been bonded to silica. The $Co(en_3)^{3+}$ formed outer-sphere complexes with the anionic nucleotides. In this separation, Mg(II) ions were used in the mobile phase since they form inner-sphere complexes with the nucleotides and thus, compete with the stationary phase for these solutes. The mechanism of retention involved, therefore, a special type of ion-pairing.

The chromatographic conditions for the ion-pair separations discussed here are given in Table 5.

TABLE 6

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOSIDE AND BASE ANALYSES USING ION EXCHANGE

Compounds* resolved	Stationary phase**	Mobile phase	Temp. (°C)	Flow-rate	Analysis time (min)	Ref. No.
Ura, Cyt, Ade, Gua, $2Me_2G$, 2MeG, 7MeG, 5MeC, 1MeA	CE: Durrum DC-1A (14 ± 2 μm, Durrum)	0.1 *M* ammonium formate (pH 4.3)	55	0.41 ml/min for 50 min then 0.79 ml/min	2 h	45
Ribo- + deoxyribonucleosides (major and rare)	CE: M-71 (10—12 μm, Beckman, Munich, G.F.R.)	0.4 *M* ammonium formate (pH 4.6)	40	7.2 ml/h	180	46
Bases and ribonucleosides	AE: Aminex A-28 (8—12 μm, Bio-Rad)	$5 \cdot 10^{-3}$ *M* citrate, $5 \cdot 10^{-3}$ *M* phosphate (pH 9.25), 55% ethanol	70	—	30	47
Bases and deoxyribonucleosides	AE: OSTION LGAT 0800 (10—12 μm, Union of Chemical and Metallurgic Manufacture, Czechoslovakia)	0.005 *M* ammonium formate (pH 4.5)	50	2 ml/h	100	48

*$2Me_2G$ = N^2,N^2-dimethylguanine; 2MeG = N^2-methylguanine; 7MeG = 7-methylguanine; 5MeC = 5-methylcytosine; 1MeA = 1-methyladenine. For other abbreviations, see Table 1.

**CE = cation exchange; AE = anion exchange.

TABLE 7

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOSIDE AND BASE ANALYSES USING REVERSED PHASE

Compounds* resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Bases, methylated and non-methylated nucleosides	μBondapak C_{18}	A: 0.02 *M* KH_2PO_4 (pH 5.6) B: methanol–water (3 : 2) Gradient linear from A to B in 87 min	Ambient	1.5	30	49
Methylated and non-methylated nucleosides	μBondapak C_{18}/Porasil	0.01 *M* $NH_4H_2PO_4$ (pH 5.0), 1% methanol	24°C	1.0	30	19
Hyp, Thy, Thd, oxypurinol, allopurinol	μBondapak C_{18}	0.025 *M* ammonium acetate (pH 5.0)	23.5°C	2.0	25	51
Ado, dAdo	μBondapak C_{18}	0.05 *M* KH_2PO_4, 10% methanol (pH 4.5)	Ambient	2.0	10	52
Ado from other nucleotides and nucleosides	μBondapak C_{18}	0.007 *F* KH_2PO_4 (pH 5.8), 10% methanol	Ambient	2.0	7	53

*For abbreviations, see Table 1.

B. Nucleosides and bases

a. Ion exchange. Ion exchange, the classical mode for the chromatography of the nucleotides, was used initially by Uziel et al. [43] and Singhal and Cohn [44] for the separation of nucleosides and bases. In 1969, Horváth et al. [34] reported the rapid separation of those compounds on pellicular cation-exchange columns. Prior to that, attempts to utilize gas chromatography for the same separations were not entirely successful. Although thin-layer and paper chromatography were widely used, quantitative analyses at the subnanomole level [34] could not be achieved.

Recently, major and rare tRNA bases [45] as well as ribo- and deoxyribonucleosides [46], have been separated by cation exchange. On the other hand, mixtures of bases and ribonucleosides [47] or deoxyribonucleosides [48] have been resolved on anion-exchange columns. As can be seen in Table 6, these analyses require the use of low flow-rates and elevated temperatures and the time needed for the separation is rather long.

b. Reversed phase. Reversed phase is at present the most commonly used liquid chromatographic mode for the separation of nucleosides and bases. The combination of microparticulate, chemically bonded packings with proper mobile phase selectivity has resulted in extremely efficient systems, capable of rapidly resolving many compounds.

Hartwick and co-workers [28, 49], in their study of UV-absorbing serum constituents, were the first to establish the chromatographic conditions necessary for the separation of bases as well as major and modified nucleosides (Fig. 3). Gehrke et al. [19] devised a separation for the analysis of ribonucleosides in urine. These analyses were especially important in view of the potential role of the methylated nucleosides as clinical markers for cancer [19, 50].

Several other assays were developed for the quantification of plasma levels of hypoxanthine, thymine, oxypurinol, thymidine [51], adenosine and deoxyadenosine [52] or serum levels of adenosine [53]. As can be seen from Table 7, these separations are fairly rapid and can be achieved at ambient temperature.

c. Ion-pairing. Since the nucleosides and bases can readily be separated by reversed phase, very few analyses have required the ion-pair mode [16, 54]. One such example is the ion-pair separation of five bases, by Ehrlich and Ehrlich [54]. Two of these, namely cytosine and 5-methylcytosine, which eluted close to the void volume, could not be resolved by reversed phases. Using LiChrosorb RP-18 (10 μm, Rheodyne) and an eluent of 5 n*M* heptanesulfonate in 2.5 n*M* potassium phosphate at pH 5.6 (25°C, flow-rate 2.0 ml/l), both bases were retained and separated from each other as well as from uracil, guanine and thymine. The analysis required only 7 min.

4. SELECTED CLINICO-BIOCHEMICAL APPLICATIONS

A. Cancer research and chemotherapy

a. Basic cancer research. Changes in the levels of the nucleosides and bases have been observed in the physiological fluids of leukemic and other cancer patients [25, 55]. In addition, changes in the levels of the methylated purines and pyrimidines may be noticeable, due to the enhanced activity of tRNA methyltransferase in certain neoplasms [19].

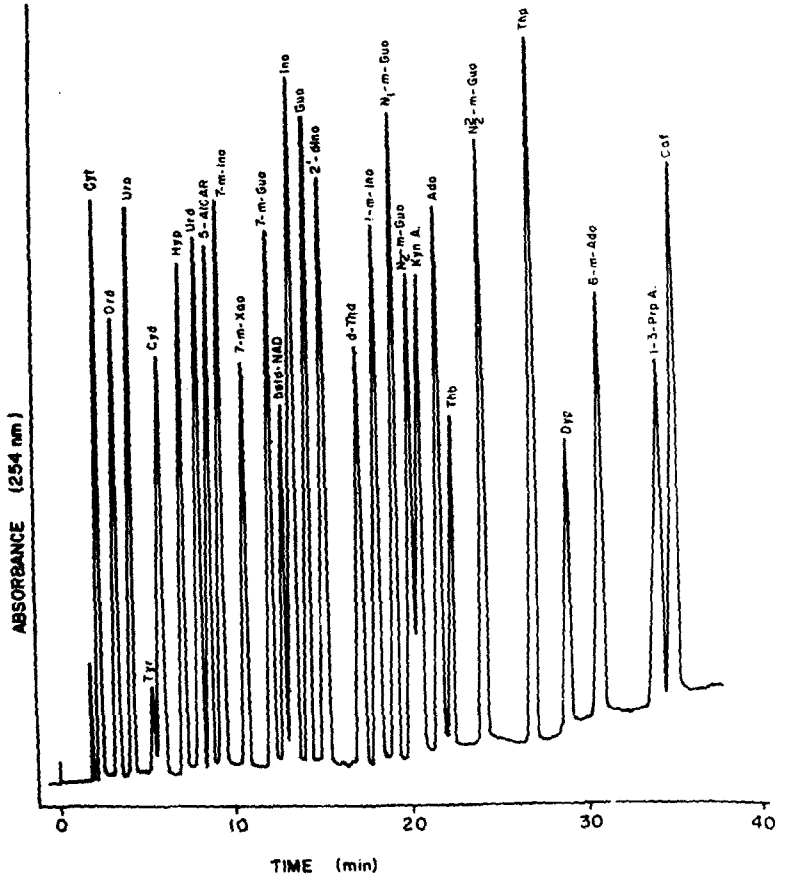

Fig. 3. Separation of 0.1—0.5 nmol of 28 nucleosides, bases, nucleotides, aromatic amino acids and metabolites. Injection volume: 40 μl of a solution $1 \cdot 10^{-5}$ mol/l in each standard. Column: chemically bonded reversed-phase (C_{18}) on 10-μm totally porous silica support. Eluents: low-strength, 0.02 mol/l KH_2PO_4 (pH 4.5); high-strength, 60% methanol. Gradient: slope 0.69%/min (0—60% methanol in 87 min), linear. Temperature, ambient; flow-rate, 1.5 ml/min. (Ref. 49.)

Gehrke et al. [19] observed elevations of 1-methylinosine, adenosine and N^2,N^2-dimethylguanosine in the urine of leukemic and breast cancer patients. In addition, the breast cancer urine chromatograms exhibited increased levels of N^2-acetylcytidine. Krstulović et al. [50] also noted the presence of 1-methylinosine and N^2-methylguanosine in the serum of some individuals with breast cancer, whereas these compounds could not be detected in the serum of normal controls (Fig. 4).

The occurrence of 7-methylguanine and O^6-methylguanine in the DNA of animals treated with carcinogens prompted Herron and Shank [56] to devise a fast chromatographic analysis for these two methylated purine bases. The assay was accomplished in 10 min on a strong cation-exchange (Partisil-10 SCX) column with an ammonium phosphate buffer eluent at pH 2.0. Both

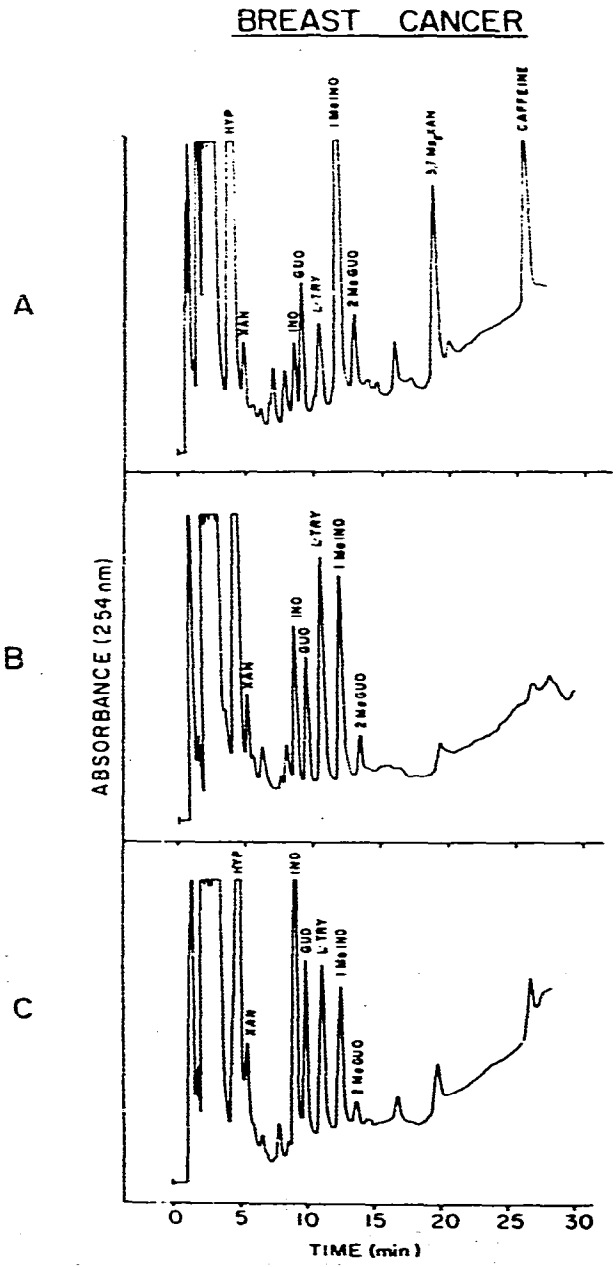

Fig. 4. (A) Chromatogram of a serum sample taken post-operatively from a non-fasting patient with breast cancer and metastasis to the bone and lymph nodes. The patient was treated by radiation and chemotherapy. (B) Chromatogram of a serum sample taken post-operatively from a non-fasting patient with breast cancer and metastasis to the bone. The patient was treated by radiation and chemotherapy. (C) Chromatogram of a serum sample taken post-operatively from a fasting patient with breast cancer with metastasis to the lung and bone. The patient was treated by radiation and chemotherapy. Chromatographic conditions for A, B and C same as in Fig. 3. (Ref. 50.)

methylated compounds can be seen in the liver DNA hydrolysate of a rat exposed to 25 μg/kg dimethylnitrosamine or the colon DNA hydrolysate of a rat treated with 163 mg/kg 1,2-dimethylhydrazine.

b. Cancer chemotherapy. In order to study the mode of action of the drugs administered during chemotherapy, assays for the drugs, their metabolites and the naturally occurring constituents in blood were needed. Brown [27], and later Scholar et al. [57], studied the effects of 6-mercaptopurine and 6-methylmercaptopurine ribonucleoside (MMPR) on Sarcoma 180 cells. A marked decrease in the adenine and guanine nucleotide pools was observed, reflecting the inhibition of their formation from IMP (Fig. 5).

In a study by Cohen et al. [13], chromatography of S-49 or murine lymphoma L5178Y cell extracts revealed that the concentrations of GTP and dGTP decreased whereas IMP increased after incubation with mycophenolic acid. These changes were attributed to the inhibition of inosinate dehydrogenase by mycophenolic acid.

Plunkett et al. [10] observed the accumulation of deazaUTP in a chromatographed extract of a brain tumor excised after intravenous infusion of deazauridine. Since other ribonucleotides were simultaneously resolved, fluctuations in the cellular concentrations of CTP could be determined and used as a sensitive indicator of the inhibitory action of deazaUTP.

Gelijkens and De Leenheer [58] optimized the separation of 5-fluorouracil (5-FU) from its deoxyribo- and ribonucleosides and nucleotides. With this new assay, the antineoplastic agent 5-FU and its metabolites in tissue extracts can readily be monitored.

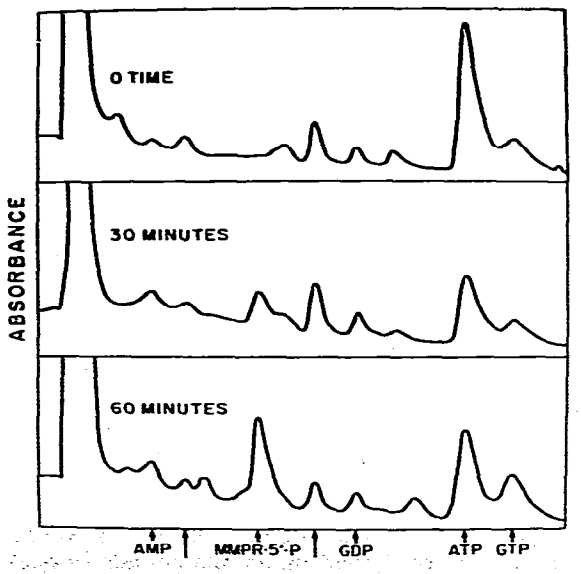

Fig. 5. Effect of 6-mercaptopurine (6-MP) and 6-methylmercaptopurine ribonucleoside (MMPR) on nucleotide patterns of Sarcoma 180 cells. Washed Sarcoma 180 cells (2 g) were incubated for 60 min with 6-MP plus MMPR. Final incubation volume was 12 ml. Extracts were prepared at 0, 30, and 60 min. Aliquots of extracts made at each time period were analyzed on a Varian Aerograph LCS-1000. Top tracing was obtained with an extract of cells prior to incubation with drug; middle and bottom tracings were from extracts of cells incubated for 30 and 60 min, respectively, with a combination of 6-MP and MMPR. (Ref. 57.)

B. Clinical studies

The levels of the nucleotides, nucleosides and bases have been quantified in the plasma, serum and urine of patients with various purine metabolic disorders. Subjects with gout and renal failure [1, 23] have been found to exhibit higher levels of hypoxanthine and xanthine than normal controls, but treatment with allopurinol reduced the oxypurine levels in the plasma of the gouty patients [1].

It was found by Bakay et al. [11] that individuals with hypoxanthine-guanine phosphoribosyltransferase deficiency had no detectable concentrations of IMP in their skin fibroblasts. Concomitantly, ATP, GTP and UTP were markedly lower than in the controls. The data reflect the lack of hypoxanthine conversion into IMP and, subsequently, other nucleotides. Whereas no free adenosine can be detected in normal human whole blood, erythrocytes or serum, this nucleoside was clearly identified in the serum of patients known to be deficient in adenosine deaminase [53] (Fig. 6). Thus, adenosine could serve as a marker for this enzyme defect, known to result in severe alterations of the immune system [59].

In order to monitor the metabolites excreted by an artificial kidney upon hemodialysis, the levels of some purines and pyrimidines were determined in the serum, dialysate and urine of a patient during a 6-h period [15]. The analytical data for the serum and dialysate were found to be essentially identical. Thus, the dialysate could be used to monitor blood composition during the time of treatment.

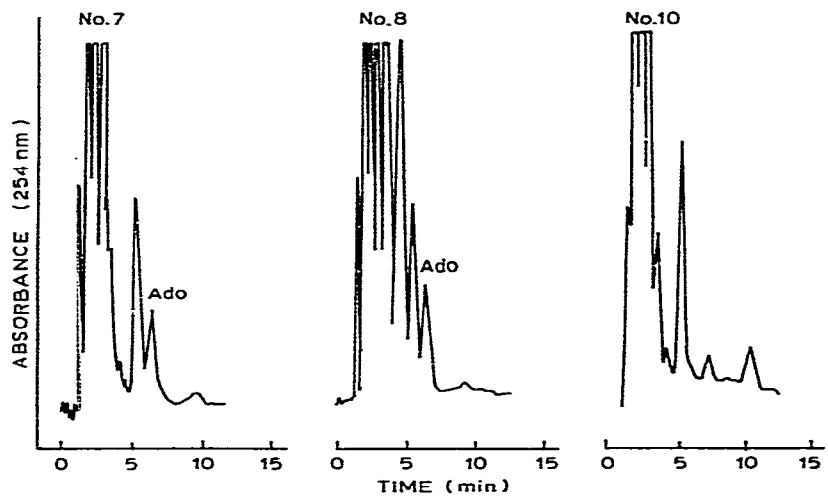

Fig. 6. Samples Nos. 7 and 8: 50 μl of serum extract from two patients suffering from adenosine deaminase deficiency; 45 and 55 picomoles are contained under the adenosine peak of sample numbers 7 and 8, respectively. Sample No. 10 shows the injection of 50 μl of serum extract from a control patient. Integrator setting, 2; column packing, μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s. Eluent, anhydrous methanol—0.007 *F* KH_2PO_4 (pH 5.8) (1:9). Flow-rate, 2.0 ml/min. (Ref. 53.)

C. Enzyme assays

Deficiencies in adenosine deaminase and purine nucleoside phosphorylase have often been associated with severe combined immunodeficiency. The early diagnosis of these deficiencies can help the clinician in administering proper therapy to control certain symptoms precipitated by the disease.

Hartwick and Brown [60] developed a rapid assay for adenosine deaminase in erythrocytes. Since the metabolism of adenosine includes the conversion of adenosine to inosine and, in turn, that of inosine to hypoxanthine, there was a need to separate adenosine, inosine and hypoxanthine from each other as well as from other red blood cell constituents. The decrease in the substrate adenosine, when added to an aliquot of erythrocyte lysate, corresponded to the increase in both inosine and hypoxanthine (Fig. 7). It is interesting to note that, using this technique, the activities of several enzymes could be monitored simultaneously.

More recently, Halfpenny and Brown [61] optimized an assay for purine nucleoside phosphorylase (PNPase) in erythrocytes. The substrate inosine, incubated with the cells, was converted into hypoxanthine. Xanthine oxidase was added to the incubation metium to prevent the accumulation of hypoxanthine which would inhibit the forward reaction of PNPase. Uric acid, hypoxanthine, xanthine and inosine were resolved for this assay with no interference from other compounds (Fig. 8).

Krstulović et al. [62] devised an assay for acid and alkaline phosphatase in serum using AMP as the substrate. After inhibition of 5'-nucleotidase by addition of Ni^{2+}, one could assay either for acid phosphatase by buffering the serum to pH 4.8, or for alkaline phosphatase by buffering to pH 9.8. Large increases in the activity of alkaline phosphatase could be observed in the serum of patients with cirrhosis or hepatitis, as compared to the normal serum.

The chromatographic conditions pertaining to all applications are listed in Table 8.

5. CONCLUSION

Whereas the nucleotide, nucleoside and base separations may be challenging and interesting to the chromatographer who seeks the understanding of retention mechanisms, they are truly essential to researchers in the clinical field. The compounds monitored and quantified may help to diagnose a disease or deficiency. The levels of nucleotides, nucleosides and bases may also reveal the mode of action of certain drugs administered in the course of chemotherapy. In addition, the rate of metabolism as well as the metabolic pathways of the drug itself may be determined. The requirements for clinical assays include rapid separation of the compounds of interest as well as reliable quantification. Although any HPLC mode can be used, reversed-phase is, at the present, the mode of choice for the analysis of nucleosides and bases. Ion-pairing is becoming increasingly popular for the separation of the nucleotides. However ion-exchange is still, to this date, the only mode which provides simultaneous analysis of the bases, nucleosides and nucleotides in samples. Since the existing separations are time-consuming, it is hoped that the ion-pairing technique will provide improved resolution as well as faster analyses.

TABLE 8

CONDITIONS FOR SELECTED APPLICATIONS

Mode*	Compounds resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
CE	Methylated purines	Partisil 10-SCX (25 cm × 4.5 mm I.D.)	0.05 *M* ammonium phosphate (pH 2.0)	–	2.0	10	56
AE	Ribonucleoside mono-, di- and triphosphates	Pellicular AX (3 m × 1.0 mm I.D.)	A: 0.015 *M* KH_2PO_4 B: 0.25 *M* KH_2PO_4 in 2.2 *M* KCl 50 ml of A in mixing chamber, B pumped into mixing chamber at a flow-rate of 5 ml/h	–	12 ml/h	80	27
AE	3-Deazauridine-5′-triphosphate other ribonucleoside triphosphates	Partisil 10-SAX	A: 0.005 *M* $NH_4H_2PO_4$ (pH 2.8) B: 0.750 *M* $NH_4H_2PO_4$ (pH 3.7) Isocratic with 40% of B for 10 min, linear to 100% B in 24 min	–	2.0	30	10
RP-IP	5-FU and metabolites	RSIL-C_{18} HL (ODS) 5-μm (15 cm × 0.32 cm)	0.02 *M* KH_2PO_4 (pH 5.0) with 5% methanol	–	0.8	10	58
RP	Ado	μBondapak C_{18}, 10 μm (25 cm × 4.6 mm I.D.)	Methanol–KH_2PO_4 (pH 5.8) (1:9)	Ambient	2.0	7	53
RP	Purine and pyrimidine bases	μBondapak C_{18}, 10 μm (60 cm × 4 mm I.D.)	A: 25 m*M* sodium acetate (pH 4.50) B: 0.1 *M* acetic acid in methanol Gradient concave in 90 min	–	1.0	80	15
RP	Adenosine deaminase	μBondapak C_{18}, 10 μm (30 cm × 4 mm I.D.)	14% methanol, 86% 0.01 *M* KH_2PO_4	Ambient	2.0	6	60

RP	Purine nucleoside phosphorylase	Partisil 5-ODS, 5 μm (25 cm × 4 mm I.D.)	0.02 *F* KH_2PO_4 (pH 4.2), 3% methanol	Ambient	2.0	7	61
RP	Acid and alkaline phosphatase	RP-8, 7 μm (25 cm × 4.6 mm I.D.)	Methanol, 0.02 *M* KH_2PO_4 (pH 5.5) (1 : 9)	Ambient	1.5	7	62

*AE = anion exchange; CE = cation exchange; RP = reversed phase; IP = ion-pairing.

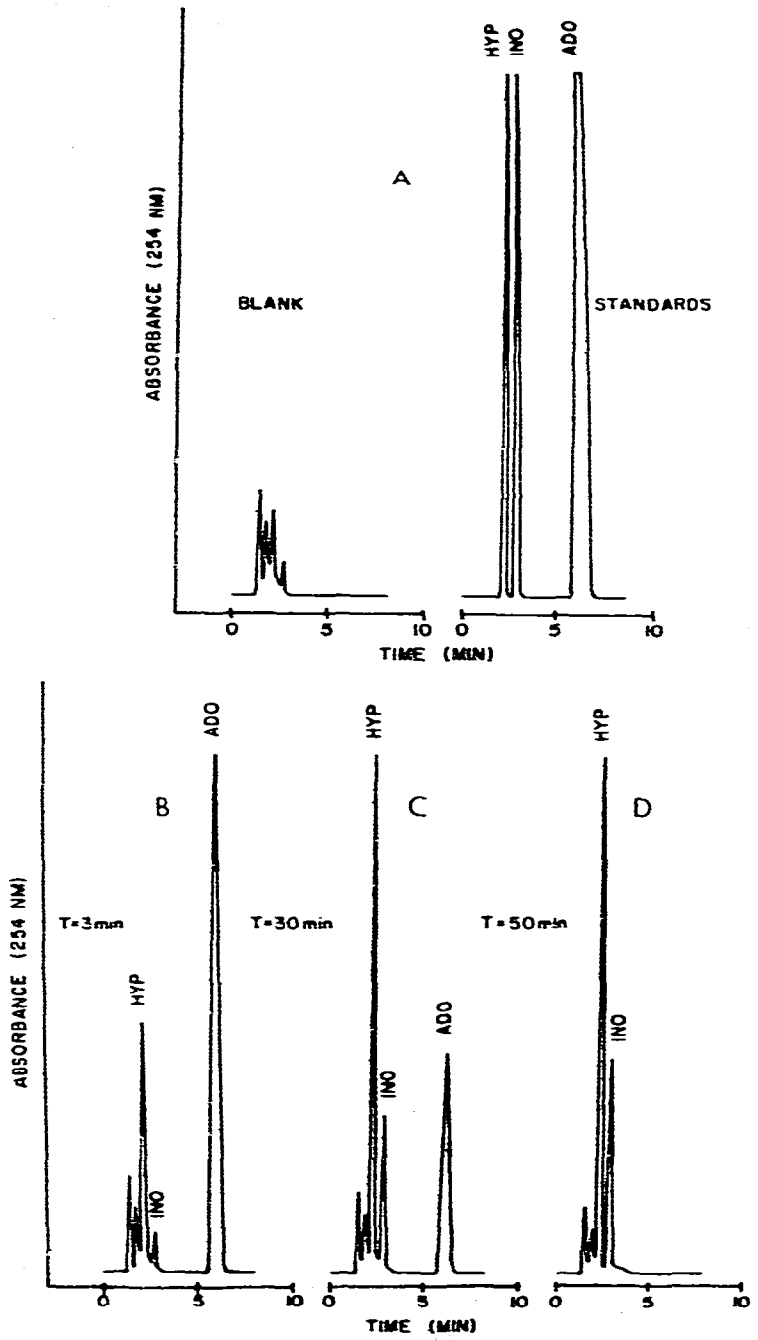

Fig. 7. The separation of the components of human erythrocytes by reversed-phase HPLC. In A a blank erythrocyte lysate is shown along with three standards: Hyp, Ino and Ado. In B, C and D, the decrease in the substrate (Ado) peak area is shown as a function of time. The chromatographic conditions are: isocratic elution, flow-rate 2.0 ml/min. Mobile phase: 86% 0.01 *F* KH_2PO_4 (pH unadjusted), 14% methanol. In each of the above chromatograms, the injection volume was 5 μl, at an attenuation of 64 on the Hewlett-Packard integrator. (Ref. 60.).

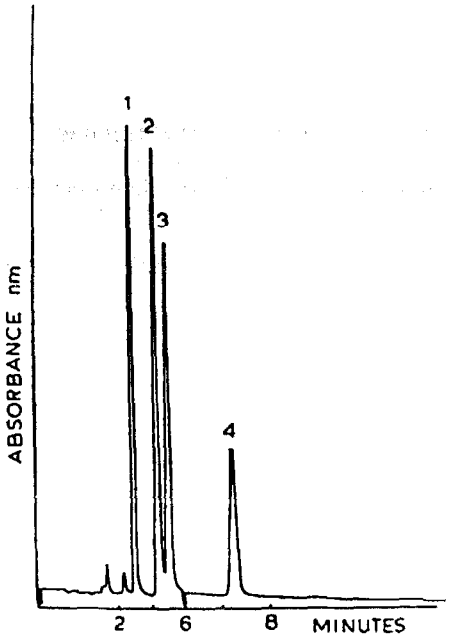

Fig. 8. Separation of the components of the reaction studied by HPLC (see text). Chromatographic conditions: isocratic elution, 2 ml/min; 0.02 *F* KH_2PO_4 (pH 4.2), 3% methanol. Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine; 4 = inosine. (Ref. 61.)

6. SUMMARY

The latest advances in the HPLC analyses of nucleotides, nucleosides and their bases in biological samples are discussed. Included are sample preparation, chromatographic procedures, identification of peaks, quantification and selected chromatographic separations for each class of compounds. The merits of the various HPLC modes for each type of separation and applications in clinical and biochemistry are presented.

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