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The Use of High-pressure Liquid Chromatography in the Analysis of the Structural Components of Nucleic Acids

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The advances in the analysis of the structural components of nucleic acids by high-pressure liquid chromatography are described and surveyed. The general ideas concerning the requirements imposed on sorbents at the present time in order to increase the efficiency of the chromatographic process are summarised. The methods and the results of the employment of effective ion-exchange sorbents in the separation and ultramicroanalysis of mixtures of the heterocyclic bases, nucleosides, nucleotides, and their derivatives are briefly considered.
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I. INTRODUCTION

The development of effective analytical methods plays a decisive role in the study of the structure of nucleic acids. The improvement of the technique of paper and thin-layer chromatography and the use of these methods in conjunction with electrophoretic and radiometric techniques made it possible to increase considerably their sensitivity and resolving capacity [1-4]. However, in view of the complexity and long time required for the application of these analytical methods, the problem of the structural analysis of nucleic acids cannot be regarded as solved.

The introduction into the ultramicroanalytical practice of a special spectrophotometric technique, the use of pumps permitting a low and controlled flow rate, and the preliminary formation of gradients in the capillary [5,6] permitted a successful solution of certain specific problems of the separation and analysis of oligonucleotides with the aid of column liquid chromatography [7]. However, the need to increase the rate of separation and the employment for this purpose of much higher pressures required a modification of the chromatographic technique. The construction in recent years of high-pressure chromatographs equipped with a reliable automatic control system and highly sensitive detecting devices as well as the appearance of new and effective sorbents greatly extended the prospects for the development of the analytical chemistry of nucleic acids [8-10], permitting the use of high-pressure liquid chromatography in the rapid ultramicroquantitative analysis of a wide variety of structural components.

Unfortunately the large-scale practical application of high-pressure liquid chromatography in structural studies on nucleic acids is to a large extent held back by the inadequate, in our view, information about the possibilities of modern ion-exchange sorbents and hence the difficulty of selecting the optimum conditions for a particular analysis. It therefore appears timely and useful to survey and give a systematic account of studies published hitherto concerning the employment of this method in the analysis of the structural components of nucleic acids and also to outline the trends in its development and further prospects for its application.

This review does not deal with the general problems of the theory of chromatography, which have been described in detail in a number of articles and monographs [8,9,11-17].

II. ION-EXCHANGE SORBENTS USED IN EFFECTIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

The theory of chromatography developed by Martin and Synge [11], Lapidus and Amundsen [12], Giddings [13,18], and a number of other investigators [19] permits the formulation of the requirements which must be met by the sorbents used in column liquid chromatography and whose fulfilment is necessary for the optimisation of the separation of the components of the mixtures analysed. We shall consider here only some of the principles underlying the theory of high-pressure liquid–liquid chromatography.

According to the Beer–Lambert law, in the region of linear working characteristics of the spectrophotometric detector (used most frequently in column liquid chromatography in the analysis of components of nucleic acids), i.e. under conditions where the detector signal is proportional to the concentration of the absorbing substance in the cell, the optical density A of the substance is specified by the equation

$$A = \lg \frac{I_0}{I} = \varepsilon c l, \qquad (1)$$

where I_0 and I are the intensities of the incident and transmitted radiation respectively, ϵ is the molar extinction coefficient of the substance, and l is the optical path length. For the Gaussian distribution, the maximum concentration of the test substance (c_{max}) in the absence of the "spreading" of the peaks in the detector cell is defined by the expression [19]

$$c_{max} = 4M/w_V\sqrt{2\pi}, \qquad (2)$$

where M is the amount of the substance in moles and W_V the width of the peak expressed in units of volume. Thus the optical density of the substance at the maximum on the distribution curve (A_{max}) is described by the equation

$$A_{max} = \varepsilon l \frac{4M}{w_V\sqrt{2\pi}}. \qquad (3)$$

The highly sensitive spectrophotometric detectors with a small cell volume (down to 8–10 μl) constructed at the present time make it possible to record extremely small (picomolar) amounts of the nucleotide [17]. Detectors of this class ensure a minimal contribution to the peak variance (up to 8%). Consequently the lower limit of the sensitivity, determined by the value of A_{max}, depends on

the "spreading" of the band of the test substance in all the remaining parts of the chromatographic system, in the first place in the chromatographic column.

The efficiency of the chromatographic column, expressed in terms of the number of theoretical plates N or the height equivalent to the theoretical plate H, is related to the peak width as follows[11]:

$$N = \frac{16t^2}{w_t^2} \quad (4)$$

or

$$H = \frac{L}{N} = \frac{Lw_t^2}{16t^2}, \quad (5)$$

where t is the retention time of the test substance on the column, w_t the peak width expressed in units of time, and L the column length (in cm).

The generalised non-equilibrium Giddings theory[12,13] makes it possible to estimate the contributions of the main components of the overall variance, which are due to the turbulent and longitudinal molecular diffusion as well as the resistance to mass transfer. The overall height equivalent to the theoretical plate, equal to the sum of the heights corresponding to the contributions of the above peak "spreading" factors, is

$$H = H_1 + H_2 + H_3, \quad (6)$$

where H_1, H_2, and H_3 are components caused respectively by the turbulent and longitudinal diffusion and the resistance to mass transfer. According to the Giddings theory[13], H_1, H_2 and H_3 can be calculated from the equations

$$H_1 = 2\lambda d_p, \quad (7)$$

$$H_2 = \frac{2\tau D_m}{v}, \quad (8)$$

$$H_3 = \frac{qrd_p^2 v}{D_s} + \frac{\omega d_p v}{D_m}, \quad (9)$$

where λ, τ, q, and ω are constants which depend on the structure of the filler as well as the form and size of the column, d_p the diameter of the filler particles v the linear rate of migration of the mobile phase, D_m the diffusion coefficient of the test substance in the mobile phase, D_s the diffusion coefficient of the test substance in the stationary phase, and r a quantity which depends on the relative rate of migration of the substance analysed and the mobile phase. As a rule, the spreading of the band of the test substance associated with longitudinal molecular diffusion is small under the conditions of the migration of the mobile phase usually employed in liquid chromatography. On the other hand, the variance of the peak due to turbulent diffusion and the resistance to mass transfer are evidently smaller, the smaller the diameter of the sorbent particles (d_p).

Thus, the direct approach to an increase of the detection limit consists in shortening the diffusion pathway of the molecules of the test substance into the sorbent particles, which can be achieved in two ways: (a) by decreasing the intrinsic size of the sorbent particles and (b) by limiting the thickness of the sorbent layer as a result of its deposition on the surface of other (inert and impermeable) particles. Both procedures are used in the modern techniques for the preparation of effective sorbents.

The development of the technology for the synthesis of polymerisation (based on styrene and divinylbenzene) fine-grain ion-exchange sorbents led to the formation of effective cation- and anion-exchange spherical microbead resins, which have been used successfully for the rapid analysis of nano- and pico-gram amounts of nucleosides and nucleotides[20,21]. For example, Fig. 1 illustrates the separation of a mixture of ribonucleosides obtained by Burtis[21] using a column with the VC-10 (×10) cation exchanger (Sondell Scientific Instruments, USA). Certain characteristics of the spherical microbead polymerisation ion-exchange sorbents used in high-pressure liquid chromatography are listed in Table 1. It is noteworthy that the use of sorbents with a very small particle diameter necessitates an appreciable increase of pressure in order to ensure the required rate of elution. This imposes in its turn a limitation on the employment of sorbents whose polymer matrix consists of a styrene–divinyl–benzene copolymer with a low (up to 7–8%) degree of cross linking. It has been shown[22] that the gel properties of such sorbents, manifested by their swelling, contraction, and compaction, as well as the decrease of their permeability, etc., give rise to virtually insurmountable technical difficulties under conditions of high-pressure chromatography.

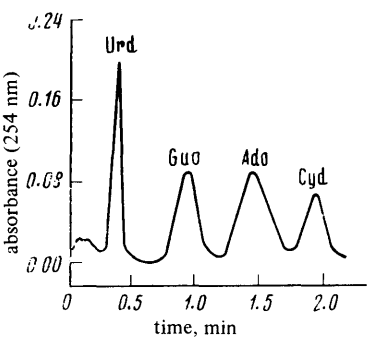


Figure 1. Separation of a mixture of ribonucleosides (300 nmoles) on a 2.4 mm × 25 cm column with a VC-10 (×10) cation exchanger in a 0.4 M $HCOONH_4$ solution (pH 4.75) at 55°C.[21]

Table 1. Certain characteristics of spherical microbead polymerisation sorbents.

Commercial name	d_p, μm	Functional groups	Capacity, mequiv. g^{-1}	Company
		Cation-exchange sorbents		
DC-X8	6—10	SO_3^-	5	Durrum Chemical Corp. USA
Aminex A-6	15—19	SO_3^-	5	Bio-Rad Laboratories, USA
Aminex A-7	7—11	SO_3^-	5	Ditto
VC-10 (×10)	7—14	SO_3^-	5	Sondell Scientific Instr.,USA
		Anion-exchange sorbents		
DA-X8	20	NR_4^+	4	Durrum Chemical Corp., USA
	6—10	NR_4^+	4	Ditto
Aminex A-27	12—15	NR_4^+	4	Bio-Rad Laboratories, USA
Aminex A-28	7—11	NR_4^+	4	Ditto

The possibility of decreasing the thickness of the sorbent bed, in which the molecules of the test substance diffuse, by converting into an ion exchanger only the surfaces of the carrier particles was first discussed in the

literature at an early stage by Pepper[23] and Weiss[24]. The successful practical utilisation of this idea in the separation of proteins on zeolite particles coated by a cation-exchange resin[25,26] stimulated the development of other surface ion-exchange sorbents and the study of their chromatographic properties[27,28]. Further development of methodological procedures involving the deposition of thin ion-exchange coatings onto glass, Teflon, and silica particles led to the creation of the so-called pellicular sorbents[29,30] and sorbents with a "controllable surface porosity".[31]

Certain characteristics of these sorbents, known under the commercial name of "Pellionex", are presented in Table 2. Sorbents of the "Pellionex" type are obtained by the copolymerisation of styrene and divinylbenzene on the surface of spherical glass particles with subsequent introduction of the appropriate functional groups[30]. In sorbents of the "Zipax" type the polymer containing functional groups is introduced into the pores of the particles[31]. Anion-exchange sorbents with a controllable surface porosity contain quaternary alkylammonium groups attached to a polyacrylic matrix and cation-exchange sorbents contain a sulphonated fluoro-polymer.

Table 2. Certain characteristics of pellicular ion-exchange sorbents and sorbents with a controllable surface porosity.

Commercial name	d_p, μm	Functional groups	Capacity, mequiv. g^{-1}	Company
Cation-exchange sorbents				
Pellionex SCX	40	SO_3^-	10	M.Reeve Angel and Co., USA
Zipax-SCX™	25—37	SO_3^-	3.2	Du Pont, Wilmington, Del., USA
Anion-exchange sorbent				
Pellionex SCX	40	NR_4^+	10	H.Reeve Angel and Co., USA
Zipax-SAX™	25—37	NR_4^+	12	Du Pont, Wilmington, Del., USA
Zipax-WAX™	25—37	NH_2	12	Ditto

The use of pellicular ion-exchangers and sorbents with a controllable surface porosity permits the rapid separation of complex mixtures of nucleotides as well as other structural components of the nucleic acid[9,32,33]. As an example, Fig. 2 presents the separation of a mixture of nucleoside mono-, di-, and tri-phosphates[29].

An undoubted advantage of surface ion-exchange sorbents is the possibility of using particles of greater size (up to 40–50 μm) in order to achieve the same efficiency of mixture separation as for fine (3–7 μm) polymerisation sorbents[9]. This permits their separation under the conditions of a high rate of elution and a considerably lower pressure. The low capacity of these sorbents[34] as well as the low mechanical strength of ion-exchange coatings, which sometimes leads to the breakdown of the surface layer during ordinary stirring of a suspension of the sorbent[9], must be regarded as their disadvantages. Furthermore, one should note the difficulty of preparing such sorbents and their high cost.

Systems with an inverted phase, known in the literature by the designations RPC-1–RPC-5,[35-38] can also be classified as surface ion-exchange sorbents. Some of them are used successfully at the present time for the separation of transport RNA[38] and the fractionation of oligo- and polynucleotides[39,40]. Silica gel or fluoro-polymer grains coated by a thin layer of liquid quaternary ammonium salts, such as trimethyloctyl- or trimethyldecyl-ammonium chlorides, are used as the stationary phase in these systems. The attainment of equilibrium between the liquid stationary and mobile phases during elution leads to a gradual "washing away" of the stationary phase. In order to increase the useful life of these sorbents, it is recommended that elution gradients and high flow rates are not used[41].

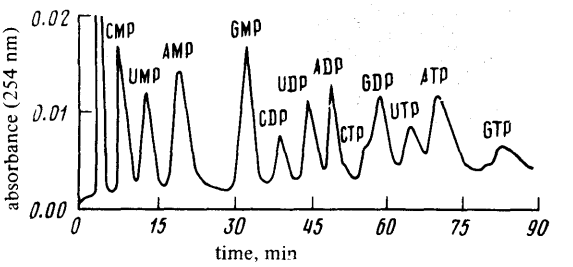


Figure 2. Separation of a mixture of nucleoside mono-, di-, and tri-phosphates (1.5–3.5 nmoles of each) on a 1 mm × 193 cm column with a Pellionex anion exchanger in a solution with a linear $HCOONH_4$ concentration gradient (0.04–1.5 M, pH 4.35) at 25°C. Rate of elution 12 ml h^{-1}; pressure 50 atm.[29]

Thus a general tendency in the development of modern high-pressure liquid chromatography is a change-over to sorbents ensuring a high rate of mass transfer and a high efficiency of the chromatographic process. A comparatively large number of effective ion-exchange sorbents, with a wide variety of structures and chromatographic properties, can be used in the analysis of the structural components of nucleic acids. The nature of the ion-exchange sorbent employed in many respects determines the conditions of the analysis. In the next section we shall consider in detail the methodological characteristics of the separation of mixtures of the most important structural components of nucleic acids under the conditions of high-pressure chromatography.

III. THE SEPARATION OF THE STRUCTURAL COMPONENTS OF NUCLEIC ACIDS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

1. Analysis of the Heterocyclic Bases

The presence in the molecules of purine and pyrimidine bases of at least one and in most cases of several functional groups capable of acid or basic dissociation permits their conversion, depending on the acidity of the medium, into anions or cations and their separation by ion-exchange chromatography. Under the conditions of high-pressure column liquid chromatography, cation-exchange sorbents are most often used for the separation of the heterocyclic bases[30,42,43], although these compounds can also be separated with the aid of anion exchangers[44].

Table 3. Conditions for the separation of the heterocyclic bases by cation-exchange chromatography on pellicular sorbents and sorbents with a controllable surface porosity.

Compn. of mixture to be separated*	Type of sorbent	Column dimensions	Eluent composition	Rate of elution, ml h^{-1}	Pressure, atm	Elution temp., °C	Separation time, min	Refs.
Ura, Gua, Ade, Cyt	Pellionex SCX	1 mm × 300.7 cm	0.02 M $NH_4H_2PO_4$ (pH 5.50)	33.4	180	68	6	30
Ura (Thy), Gua, Cyt, Ade	Pellionex SCX	1 mm × 300 cm	0.05 M $NH_4H_2PO_4$ (pH 2.55)	40	115—143	70	10	42
Ura, Gua, Cyt, Ade	Pellionex SCX	1 mm × 250 cm	0.025 M $NH_4H_2PO_4$ (pH 4.40)	36	200	70	5	42
Ura, Hyp, Gua, Cyt, Ade, 6-MePu	Zipax-SCX™	2.1 mm × 100 cm	0.1 *N* HNO_3	120	53	63	15	43

*The compounds are quoted in order of increasing retention time on the column. The following abbreviations are used: Ura = uranil; Gua = guanine; Ade = adenine; Cyt = cytosine; Thy = thymine; Hyp = hypoxanthine; 6-MePu = 6-methylpurine.

It has been shown[30,42,43] that the effective separation of standard mixtures of heterocyclic bases can be achieved after 5–10 min using sorbents of the Pellionex or Zipax-SCX™ type. The detailed conditions for the separation of mixtures of heterocyclic bases are presented in Table 3.

In view of the comparatively small differences between the positive charges of the heterocyclic bases, they are usually separated under isocratic† elution conditions. The most satisfactory separation of the four nucleic acid bases on columns with a cation exchanger of the Pellionex type is achieved on elution with a 0.02 M $NH_4H_2PO_4$ solution (pH 5.2–5.5) at 68°C[30] (Fig. 3).

The behaviour of compounds in the course of chromatographic analysis is known to be determined both by the charge of their molecules and by their non-ionic affinity for the sorbent. Analysis of the elution sequence of the heterocyclic bases when the latter are separated on columns with sorbents of the Pellionex and "Zipax-SCX™" type (Table 3) permits the conclusion that ionic interactions are in this instance expressed more strongly than in the case where the usual polymerisation cation-exchange sorbents of the type Dowex-50, Amberlite IRC-50, etc. are used[45]. Indeed, when the eluent is a buffer at pH 5.5, the elution sequence of Uracil, guanine, (pK'_a = 3.3), adenine (pK'_a = 4.2), and cytosine (pK'_a = 4.6) corresponds to an increase of the pK_a values for the bases (i.e. to an increase of the positive charge of the molecules[46]). On the other hand, at lower pH values the order in which adenine and cytosine are eluted is reversed[42,43]. The data obtained by Horvath and Lipsky[30] also indicate an anomalous behaviour of guanine on columns with a Pellionex cation exchanger at pH 5.2 ("twinning" of the peak corresponding to guanine was observed).

It is apparently impossible to explain these phenomena from the standpoint of the non-polar interactions of the molecules of the test substance with the sorbent particles, because the non-polar affinity is almost independent of the pH of the medium[45]. The authors' suggestion that the anomalous behaviour of the test compounds on pellicular sorbents may be associated with kinetic effects in the course of the attainment of the sorption–desorption equilibrium involving the molecules of the test substance in the stationary and mobile phases also appears unconvincing[30].

†Isocratic elution is elution with a solution at a constant ionic strength.

It is noteworthy that the use of the Zipax-SCV™ cation-exchange sorbent with a controllable surface porosity permits the elution of bases with a solution having a lower ionic strength (even at a lower pH of the eluent), because of its lower capacity (Table 2).[43]

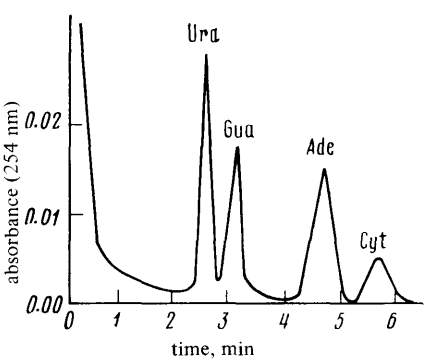


Figure 3. Separation of a mixture of heterocyclic bases (0.8 nmole of each) on a 1 mm × 300.7 cm column with a Pellionex cation exchanger in a 0.02 M $NH_3H_2PO_4$ solution (pH 5.5) at 68°C. Rate of elution 33.4 ml h^{-1}; pressure 177 atm.[30]

The possibility of employing spherical microbead polarisation cation-exchange resins under the conditions of high-pressure chromatography for the analysis of the heterocyclic bases was first demonstrated[47] in the determination of the amount of DNA in various tissues on the basis of their thymine context. The separation of the mixture of the heterocyclic bases formed as a result of the active hydrolysis of DNA has been achieved[48] on a column with the Aminex A-7 sorbent in 1 M NaH_2PO_4 (pH 3.4). Under these conditions, the non-ionic interactions of the mixture components with the organic matrix of the sorbent were suppressed by the increased elution temperature and the addition of alcohol to the solution. The necessity for the separation of mixtures of the heterocyclic bases arises fairly frequently in the course of a whole series of biochemical studies, particularly in the determination of the composition of RNA and DNA, in the elucidation of the

possible reaction pathways of free nucleotides *in vivo*, in the study of the mechanism of the action of certain enzymes etc., and in all cases high-pressure ion-exchange chromatography is as a rule one of the most effective analytical methods[8,9,49,50].

2. Analysis of Nucleosides

Numerous studies have been devoted to the separation and analysis of mixtures of nucleosides and their derivatives under the conditions of high-pressure ion-exchange chromatography[9,21,30,42]. This is associated primarily with the increased availability of highly purified enzymes in recent years, which permits the hydrolysis under mild conditions of ribo- and deoxyribo-nucleotide chains to nucleosides, as a result of which many problems involved in the study of the structures of nucleic acids can be solved at the level of the analysis of the nucleosides.

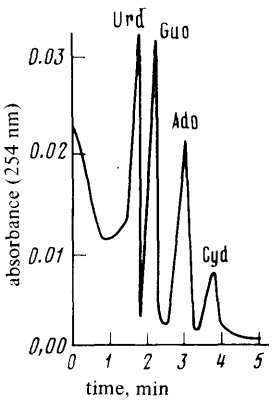


Figure 4. Separation of a mixture of nucleosides (approximately 0.3 of a nmole of each) on a 1 mm × 151.7 cm column with a Pellionex cation exchanger in a 0.02 M $NH_4H_2PO_4$ solution (pH 5.5) at 24°C. Rate of elution 32.5 ml h^{-1}; pressure 38 atm.[22]

Table 4 summarises the literature data for different types of sorbents and separation conditions most frequently used in the analysis of nucleosides. Evidently both spherical microbead polymerisation cation-exchange resins[21] and pellicular cation exchangers of the Pellionex[22,41] type as well as Zipax-SCX™ sorbents with a controlled surface porosity[9] can be used for the separation of nucleosides and many of their derivatives under the conditions of high-pressure liquid chromatography. However, a more detailed analysis of the results of the separation obtained using the above sorbents suggests that preference should be given to spherical microbead polymerisation resins, because the sorption of uridine and thymidine on columns with sorbents of the Pellionex and Zipax-SCX™ types under the conditions of optimum separation of the nucleoside peaks is extremely low, as noted by Horvath[9]. Because of this, the above nucleosides are eluted in a very narrow band and their peaks are as a rule superimposed on the "injection peak", which normally accompanies the introduction of the analysed solution into the column (Fig. 4). This hinders and sometimes altogether precludes a correct interpretation of the results of the separation process. On the other hand, the use of spherical microbead cation-exchange resins, whose polymeric matrix in all probability ensures a fairly intense sorption, makes it possible to overcome these difficulties. The best results in the separation of nucleosides and many of their derivatives were obtained by Burtis[21] on columns with the finest DC-x8 (Fig. 1) and VC-10 cation-exchange resins. Burtis also confirmed the theoretical conclusion that the maximum efficiency of the separation can be achieved when the column is filled with finer particles (Table 5).

Table 5. The efficiency of columns with spherical microbead cation-exchange polymerisation sorbents in the separation of nucleosides in 0.4 M ammonium formate solution (pH 4.55).[42]

Sorbent	d_p, μm	Column dimensions	Temp., °C	Rate of elution		Pressure, atm	N	H, mm
				ml h^{-1}	cm s^{-1}			
DC-×8	3—7	2.4 mm × 25 cm	55	40	0.61	326	1042	0.24
Aminex A-7	7—10	2.4 mm × 25 cm	55	35	0.54	300	806	0.31
VC-10 (×10)	7—14	2.4 mm × 25 cm	85	85	1.30	334	338	0.74

Table 4. Conditions for the separation of nucleosides by high-pressure cation-exchange chromatography.

Composition of the mixture to be separated*	Type of sorbent	Column dimensions	Eluent composition	Rate of elution, ml h^{-1}	Pressure, atm	Elution temp., °C	Separation time, min	Refs.
Urd, Guo, Ado, Cyd	Pellionex SCX	1 mm × 151.7 cm	0.02 M $NH_4H_2PO_4$ (pH 5.60)	25.5	140	39	4	30
dThd, dGuo, dAdo, dCyd	Aminex A-7	2.4 mm × 25 cm	2.4 M $HCOONH_4$ (pH 4.55)	10	70	55	20	42
Urd, Guo, Ado, Cyd	Pellionex SCX	1 mm × 300 cm	0.012 M $NH_4H_2PO_4$ (pH 3.50)	33	150—170	50	16	42
Urd, Ino, Guo, Ado, Cyd	DC-×8	2.4 mm × 25 cm	0.4 M $HCOONH_4$ (pH 4.75)	40	340	55	4	9
Urd, Guo, Ado, Cyd	VC-10 (×10) (17-14 μm)	2.4 mm × 24 cm	0.4 M $HCOONH_4$ (pH 4.50)	50	215	75	7	21
Urd, Guo, Ado, Cyd	VC-10 (×10) (7-10 μm)	2.4 mm × 25 cm	0.4 M $HCOONH_4$ (pH 4.50)	50	330	75	4	21

*The compounds are quoted in order of increasing retention time on the column. The following abbreviations are used: Urd = uridine; dAdo = deoxyadenosine; dGuo = deoxyguanosine; dThd = deoxythymidine; dCyd = deoxycytidine; Ino = inosine.

Spherical microbead polymerisation cation-exchange sorbents have been used successfully in the analysis of the enzymatic hydrolysates of many RNA specimens in order to elucidate their nucleoside composition[9,21,42] and also for the investigation of the content of various minor nucleosides in DNA[51] and individual transport RNA[34,50] preparations.

Mixtures containing simultaneously ribo- and deoxyribo-nucleosides can be separated comparatively rapidly on columns with the Aminex A-7 sorbent proposed by Duch and Laskowski[52]. The difference between the retention times of ribose and deoxyribose derivatives can be most probably accounted for by the secondary interactions of the nucleosides with the sorbents (by hydrogen bonding, etc.) rather than ionic interactions. The method has been used successfully to determine the impurity amounts (down to 0.2–0.4%) of RNA in DNA preparations after the enzymatic degradation of the polymeric molecules to nucleosides[52].

The traditional procedure for the preparation of carbohydrate-borate complexes, proposed as early as 1951[53] and developed in detail subsequently[45,54], has also been used successfully to separate ribo- and deoxyribo-nucleosides under the conditions of effective liquid chromatography. By combining ion-exclusion and partition chromatography in columns with the Aminex-A-6 sorbent using a 0.1 M ammonium borate solution as the eluent (pH 7.4), it is possible to separate ribo- and deoxyribo-nucleosides in view of the capacity of the former for complex formation with the borate ions of the eluent. The ribose derivatives converted into anions under these conditions are "expelled" from the negatively charged stationary phase.

Ion-exclusion chromatography has been used recently on an increasing scale for the separation of mixtures of nucleosides. The principle of this method has been frequently discussed in the literature[55,56]. The difference between the negative charges of the molecules as well as the different degrees of their non-specific sorption permit a comparatively rapid separation in an alkaline medium (pH 9.5) of complex mixtures of nucleosides on columns with Aminex A-6 cation-exchange resins[57,58].

The use of finer polymerisation resins and higher flow rates in ion-exclusion chromatography apparently makes it possible to increase greatly the efficiency of the method, which thus leads to considerable prospects for the employment of the high-pressure chromatographic technique.

3. Analysis of Mononucleotides and Their Derivatives

Anion-exchange sorbents are usually employed for the separation of mixtures of mononucleotides, because these compounds and the majority of their derivatives exist in the anionic form as a result of the acid dissociation of the phosphate groups ($pK_a^1 = 1$; $pK_a^2 = 6-7$[59]) over the entire range of the pH of the eluents normally used in ion-exchange chromatography. The data presented in Table 6 show that the Pellionex anion exchangers and the Zipax-SAX^{TM} sorbents are used most extensively for the analysis of these compounds under the conditions of high-pressure chromatography. The employment for these purposes of columns with spherical microbead polymerisation anion-exchange resins is limited[60,61], which in our view is due to the breakdown of the column packing in consequence of

Table 6. Conditions for the separation of nucleotides and their derivatives by high-pressure anion-exchange chromatography.

Compn. of mixture to be separated*	Type of sorbent	Column dimensions	Eluent compn.	Rate of elution, ml h^{-1}	Elution temp., °C	Pressure, atm	Separation time	Refs.
P-2′-Cyd, P-3′-Cyd, P-2′-Urd, P-3′-Urd, P-2′-Ado, P-3′-Ado, P-2′-Guo, P-3′-Guo	Dowex-1 × 8 (~ 10 μm)	0.25 mm × 75 cm	(0.05—1.0 *M*) CH_3COONa (pH 4.40) **	60	45	285	240	60
AMP, ADP, ATP	Pellionex	1 mm × 300 cm	0.75 *M* KH_2PO_4 (pH 4.2) **	55	80	186—214	4	42
CMP, UMP, AMP, GMP, CDP, UDP, ADP, GDP, CTP, UTP, ATP, CTP	Pellionex	1 mm × 193 cm	(0.04—1.5 *M*) $HCOONH_4$ (pH 4.35) **	12	25	54	90	30
CMP, UMP, AMP, IMP, GMP, XMP	Pellionex	1 mm × 300 cm	0.01 *M* KH_2PO_4 (pH 3.25)—1 *M* KH_2PO_4 (pH 4.20) **	15	70	200	55	42
dCMP, dTMP, dAMP, dGMP	Pellionex	1 mm × 300 cm	0.01 *M* KH_2PO_4 (pH 3.25)—1 *M* KH_2PO_4 (pH 4.20) **	57	80	170—200	10	42
P-2′-Cyd, P-3′-Cyd, P-2′-Urd, P-3′-Urd P-2′-Ado, P-3′-Ado, P-2′-Guo, P-3′-Guo	Pellionex	1 mm × 300 cm	0.01 *M* KH_2PO_4 (pH 3.35)—1 *M* KH_2PO_4 (pH 4.20) **	48	75	207	30	9
CMP, UMP, AMP, GMP	Zipax-SAX^{TM}	2.1 mm × 100 cm	0.006 *M* H_3PO_4, 0.002 *M* KH_2PO_4 (pH 3.75) ***	114.6	60	64	7	43
dCMP, dTMP, dAMP, dGMP	Zipax-SAX^{TM}	1 mm × 100 cm	0.008 *M* KH_2PO_4 (pH 4.40) ***	2.5	20	150	70	62

*The compounds are quoted in order of increasing retention times on the column. The following abbreviations are used: P-2′(3′)-Cyd = cytididine 2′(3′)-phosphate; P-2′(3′)-Urd = uridine 2′(3′)-phosphate; P-2′(3′)-Ado = adenosine 2′(3′)-phosphate; P-2′(3′)-Guo = guanosine 2′(3′)-phosphate; dCMP = deoxyribocytidine 5′-phosphate; dTMP = deoxyribothymidine 5′-phosphate; UMP = uridine 5′-phosphate; dAMP = deoxyriboadenosine 5′-phosphate; dGMP = deoxyriboguanosine 5′-phosphate; IMP = inosine 5′-phosphate; XMP = xanthosine 5′-phosphate.

**Gradient elution.

***Isocratic elution.

the change in the swelling of the sorbent. In addition, a change in the composition of the eluting buffer normally leads to the compaction of the polymerisation sorbents, which is extremely undesirable under the conditions of high-pressure chromatography.

Attention should be drawn to the difference between the conditions for the separation of mixtures of nucleotides on various sorbents (Table 6), which may be caused by the different chemical structures of the sorbents themselves. Thus the separation of certain mixtures (for example of ribonucleotides, deoxyribonucleotides, etc.) under the conditions of isocratic elution[43,62] can be achieved on columns with the Zipax-SAX^{TM} ion exchanger. In particular, an effective and rapid separation of nucleoside 5′-phosphates has been achieved on columns with this sorbent using a 0.002 M KH_2PO_4 solution at pH 3.75 (Fig. 5). The use of a Pellionex carrier for this purpose requires the application of gradient elution[9,42]. Changes in the pH of the eluent are also frequently used together with changes in concentration in order to improve the resolution and shape of the peaks[9,42,43].

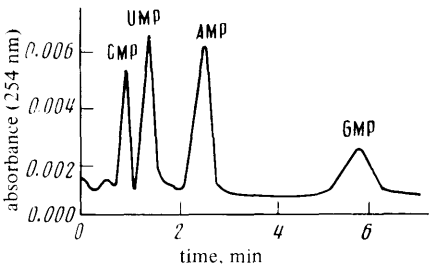


Figure 5. Separation of a mixture of nucleoside 5′-phosphates on a 2.1 mm × 100 cm column with the Zipax SAX^{TM} sorbent in a 0.002 M KH_2PO_4 solution (pH 3.75) at 60°C. Rate of elution 114.6 ml h^{-1}; pressure 64 atm.[43]

The position of the phosphate group in the nucleotide molecule influences the degree of protonation of the heterocyclic base[59] and hence the overall charge of the molecule and permits thereby the separation of isomeric nucleoside phosphates[9]. The conditions indicated in Fig. 6 for the separation of a mixture of nucleoside 2′,3′-phosphates have been used to analyse alkaline hydrolysates of RNA.[53]

The technique of high-pressure anion-exchange chromatography and the use of effective sorbents permit comparatively rapid separation of mono-, di-, and triphosphates of ribo- and deoxyribo-nucleosides[9,29,54] (see also Fig. 7), which require elution for many hours under the conditions of the usual chromatography[65]. Table 7 presents the retention times of nucleoside mono-, di-, and triphosphates as well as a number of their derivatives obtained by Brown[66] in chromatography on a Pellionex anion exchanger, which are extremely useful for the interpretation of chromatograms obtained in the separation of mixtures of free nucleotides and their derivatives from various extracts.

The possibility of rapid analysis of picomolar amounts of adenosine 5′-phosphate, adenosine 3,5′-cyclic phosphate, and adenosine 5′-triphosphate on columns with a Pellionex anion exchanger has been studied by a number of investigators in order to determine the activities of adenylate cyclase and adenosine 3′,5′-cyclic phosphate diesterase, which catalyse the reactions of the corresponding adenylic acid derivatives in tissue cells[67-69].

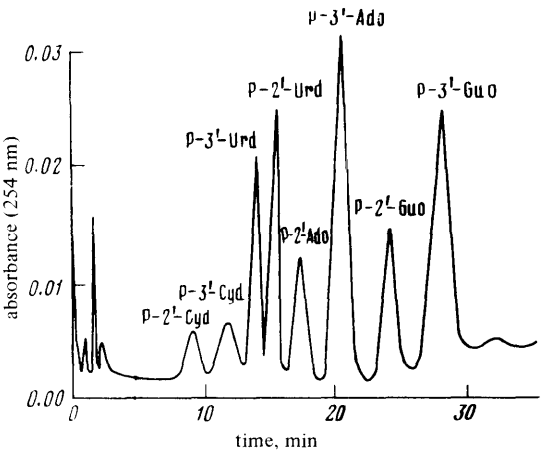


Figure 6. Separation of a mixture of nucleoside 2′,3′-phosphates (approximately 1 nmole of each) on a 1 mm × 300 cm column with a Pellionex anion exchanger in a solution with a linear KH_2PO_4 concentration gradient (0.01 M, pH 3.35–1.0 M, pH 4.20) at 75°C. Rate of elution 48 ml h^{-1}; pressure 207 atm.[64]

Table 7. The retention times of nucleotides and their derivatives in chromatography on a 1 mm × 300 cm column with a Pellionex anion exchanger*.

Compound**	Time, min	Compound**	Time, min	Compound**	Time, min
CMP	10	NAD+	8.5	CTP	46
UMP	12	UDP-Glc	24	UTP	49.5
TMP	14	CDP	29	TTP	54
IMP	18	UDP	31	ATP	60
AMP	19	TDP	33	GTP	65
GMP	23	ADP	38		
6Me-sPuoMP	27	NADH	39		
sGMP	32	GDP	43		
XMP	34	UDP-GlcCOOH	44		

* Chromatographic conditions[66]: elution in a linear phosphate buffer gradient [0.015 M KH_2PO_4 (pH 3.25)–0.25 mm KH_2PO_4 (pH 4.20), 2.2 M KCl] at 75°C. Rate of elution 12 ml h^{-1}.

** The following abbreviations are used: 6Me–sPuoMP = 6-methylthioribosylpurine 5′-phosphate; sGMP = mercaptoguanosine 5′-phosphate; NAD^+, NADH = nicotinamide-adenine dinucleotide (oxidised and reduced); UDP-Glc = uridine diphosphate-glucose; UDP-GlcCOOH = uridine phosphate-glucuronic acid.

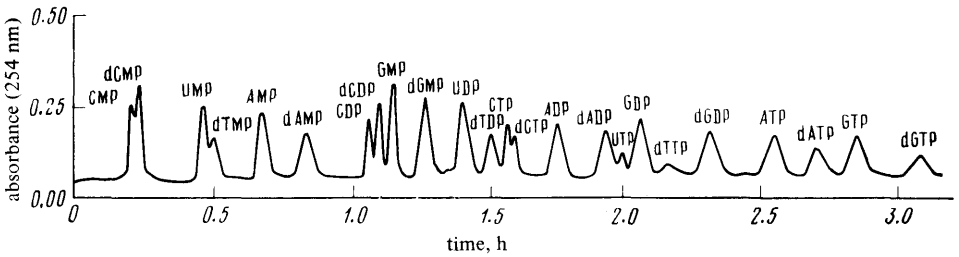


Figure 7. Separation of a mixture of ribo- and deoxyribo-nucleoside phosphates on a 0.62 mm × 20 cm column with the Aminex A-28 sorbent in a solution with a sodium nitrate concentration gradient (pH 8.6) at 70°C. Rate of elution 36 ml h^{-1}; pressure 15 atm.[64]

The successful separation of a mixture of nucleosides and nucleotides on columns with the Aminex A 28 polymerisation anion exchanger[48] (Fig. 8) permits rapid analysis of the oligonucleotide end groups, which is one of the essential stages in the study of the primary structure of the fragments of native nucleic acids.

Numerous studies on the content of the mononucleotides and their derivatives in various extracts in connection with biochemical and medicobiological research[70-73] also indicate the unique possibilities of high-pressure liquid chromatography, with which none of the available analytical methods can compete nowadays in this field.

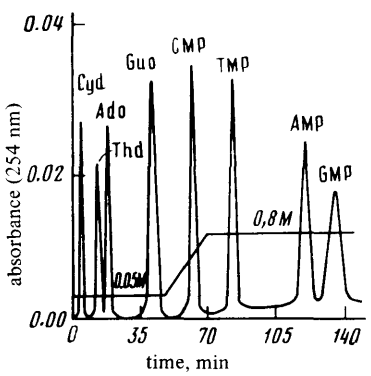


Figure 8. Separation of a mixture of deoxyribonucleosides and deoxyribonucleoside 5′-phosphates on a 1.8 mm × 50 cm column with the Aminex A-28 sorbent in a solution with a concentration gradient of a phosphate–borate buffer (0.05–0.8 M, pH 7.8) at 60°C. Rate of elution 25 ml h^{-1}; pressure 220 atm.[48]

4. Chromatography of Oligo- and Poly-nucleotides

Highly efficient separation of oligo- and poly-nucleotides with respect to both chain length and nucleotide composition is one of the urgent problems in the analytical chemistry of nucleic acids. The possibilities of high-pressure liquid chromatography as regards the solution of this problem have so far been inadequately investigated. The choice of sorbents for the separation of oligo- and poly-nucleotides whose behaviour in chromatography is determined not only by the structure of heterocyclic bases, the nature of the sugar residues, and the overall charge of the molecule but also by the molecular weight and characteristics of the steric interaction with the surface of the ion exchanger[77,78], constitutes a fairly complex problem. In this connection the permeability of sorbent particles to large molecules assumes particular importance. Thus the spherical microbead polymerisation anion exchangers with a high content of cross-links (up to 8–10%), used in high-pressure liquid chromatography are either altogether impermeable to polyanions with a high molecular weight or their permeability is extremely low owing to the low rate of diffusion of these compounds into the sorbent particles. On the other hand, as mentioned above, sorbents with a smaller content of cross-links are not used in high-pressure liquid chromatography in view of their more pronounced gel properties. They can be employed only as an ion-exchange film coating in the so-called pellicular sorbents[9]. However, the possibility of using such sorbents in the practical chromatography of oligonucleotides has been scarcely elucidated. Pellicular anion exchangers have so far been used only for the separation of dinucleotides[9,42,64].

The RPC-3 and RPC-5 anion-exchange systems with an inverted phase are used most frequently for the analysis of oligo- and poly-nucleotides[35-38]. These sorbents, first introduced into liquid chromatography by Kelmers and coworkers[35] for the fractionation of transfer RNA, are nowadays used successfully also for the separation of oligo- and poly-nucleotides[39,40]. Their chromatographic characteristics depend on the chemical nature of the liquid stationary phase, which usually consists of liquid tetraalkylammonium salts, as well as the structure of the particles which serve as the solid support.

The solid supports in the RPC-3 and RPC-4 systems consist of silica or silica gel particles. To ensure the firm binding of the liquid quaternised amine to the surface of such particles, the latter are silylated, i.e. treated with trialkyl- or triarylchlorosilanes[37,39]. The data obtained by Holton et al.[39] suggest that the use of chlorotriphenylsilane for this purpose is preferable, because it improves considerably the chromatographic properties of the sorbent. Holton et al.[39] were able to achieve with the aid of the sorbents the separation of thymidylic acid oligonucleotides under the conditions of isocratic elution with a 0.5 M solution of an ammonia–formate buffer (pH 5.25). The RPC-5 system, where fluoro-polymer particles are used as the solid support, also proved to be effective for the

fractionation of oligo- and poly-nucleotides with respect to their chain length[40]. Use of the system enabled the authors[40] to achieve the separation of a 60-component mixture of homologous nucleotides having the general formula $(Ap)_xU$ ($x = 1, 2, 3, \ldots$).

The lack of information concerning the separation on these sorbents of mixtures of oligo- and poly-nucleotides with a more complex structure precludes so far an assessment of the prospects and limits of their employment. There is no doubt that the capacity of certain oligonucleotides for self aggregation in aqueous salt solutions[79] and the associated enhancement of the role of secondary non-ionic interaction create certain difficulties in their chromatography on sorbents of this kind. On the other kind, the impossibility of employing under the conditions of high-pressure liquid–liquid chromatography of organic compounds such as urea, dioxan, etc. (normally used to suppress secondary interactions of this kind[80,81]), owing to the considerable increase of the viscosity of the mobile phase and the increase of the solubility in the latter of the liquid stationary phase, aggravate these difficulties still further.

In this connection the study of the possibility of using for the separation of mixtures of oligo- and poly-nucleotides of coarsely porous sorbents with a bound ion-exchange phase[41] in which the hydrocarbon groups of amines are attached to silica gel particles via strong covalent bonds, which have become available in recent years, appears to be very promising. These sorbents include Partisil-10 SAX (Whatman, England) and Lichrosorb NH_3 (E.Merk, German Federal Republic). While possessing all the advantages of systems with an inverted phase, they permit an extensive variation of the composition of the eluates, extending thereby appreciably the scope of their applications.

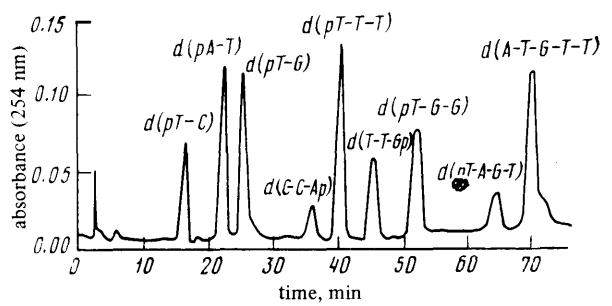


Figure 9. Chromatography of oligodeoxyribonucleotides on a 2 mm × 25 cm column filled with silica gel (particle size approximately 10 μm) with covalently attached hydrocarbon groups of primary amines in a solution with a linear phosphate buffer concentration gradient (0.10 M KH_2PO_4, pH 3.30–0.75 M KH_2PO_4, pH 4.25) at room temperature. Rate of elution 60 ml h^{-1}; pressure 180 atm.[81]

Data obtained by Aukati et al.[81] have shown that columns containing silica gel with grafted aminoalkyl groups can be used successfully for the fractionation of olinucleotides with respect to both their length and composition (Fig. 9). The stability of the stationary phase in such columns with respect to organic solvents and their stability over a wide pH range (1–8.5) ensure a satisfactory reproducibility of the analytical results and a long lifetime of the columns[82].

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Summarising the data discussed in this review, one must conclude that the application of modern high-pressure ion-exchange chromatography permits the successful separation of ultramicroanalytical amounts of a very great variety (as regards complexity) of mixtures of the structural components of nucleic acids. The commercial availability of the necessary chromatographic apparatus and ion-exchange sorbents ensuring a high rate of mass transfer and the associated efficiency of the chromatographic process permit the employment of the high-pressure liquid chromatographic technique in the solution of a very large number of problems in research on the composition, structure, and functional role of nucleic acids. One may hope that further investigation of the possibilities of the sorbents already available and the appearance of new ion-exchange materials will eventually permit a further extension of the limits of applicability of the high-pressure liquid–liquid chromatographic technique, whose efficiency is in many instances comparable to that of gas chromatography.

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