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Review

Perspectives on analyses of nucleic acid constituents: the basis of genomics

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Abstract

The recent mapping of the human genome was a tremendous achievement made possible to a large degree by the development of analytical methods for sequencing purine and pyrimidine bases in nucleic acids. In the last 3 decades, the number of analyses of nucleic acids and their constituents by HPLC and capillary electrophoresis (CE) has exploded. These techniques have been used not only for genomics, but also for the determination of free nucleotides, nucleosides and their bases in body fluids and tissues. Although a large number of HPLC and CE papers have been published on nucleic acid constituent applications, relatively little has been written on the mechanisms of the separations. However, to optimize analytical conditions knowledgeably and rapidly, it is important to know why and how these separations occur and the factors that affect them. The HPLC methods for the analysis of nucleic acid constituents and the information available on some of the mechanisms of separation of nucleotides, nucleosides and their bases, as well as the analysis of these compounds by CE and the factors that affect these separations are discussed.

Keywords: Reviews; Nucleic acids; Nucleotides; Nucleosides; Nucleobases

Contents

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1. Introduction

One of the most exciting research projects of the twentieth century was the deciphering of the code of the human genome. As E.S. Lander, director of the Whitehead Institute, Center for Genomic Research at MIT said in essence:

> *Genomics . . . aims to produce biology's periodic table – not 100 elements, but 100,000 genes.*

(see Ref. [1]).

A gene consists of double stranded deoxyribonucleic acid (DNA), which contains the genetic or hereditary material of an organism. Sequencing the genes in the human genome was a tremendous achievement made possible by analytical methods for identifying the sequences of the purine and pyrimidine compounds that compose our DNA. However, before the sequencing could be done, reliable methods for the separation and identification of the nucleic acid constituents, the nucleotides, nucleosides and their bases, had to be developed. The techniques that originally were used, and are still in use today, are HPLC and gel electrophoresis. In addition, capillary electrophoresis is being used more frequently, as it is faster than many gel electrophoresis methods. These techniques have been used not only in genomics, but also in the determination of nucleic acid constituents in body fluids and cells.

There are two types of nucleic acids, DNA and ribonucleic acid (RNA). Each is a polymer chain with similar monomer units connected by covalent bonds. Each monomer unit contains a five-carbon sugar and a triphosphate group. The connection between successive monomer units in nucleic acids is through a phosphate residue attached to the hydroxyl on the 5′ carbon of one unit and the 3′ hydroxyl of the next one; thus a phosphodiester link is formed between the two sugar residues which make up the backbone of the molecule (Fig. 1).

Each monomer in the chain also carries a base group, which is attached to the 1′ carbon of the sugar. There are two types of bases, purines and pyrimidines. DNA has two pyrimidines, cytosine and thymine, and two purines, adenine and guanine whereas RNA contains the same bases except that uracil replaces thymine. Although only four bases compose DNA, the specific sequences of these nucleic acids are responsible for gene expression (Figs. 2 and 3). Through gene expression, enzymes and proteins are produced which code for hereditary information including genetic diseases. Modification of purines and pyrimidines has resulted in extremely effective drugs. Studies to monitor the metabolism of

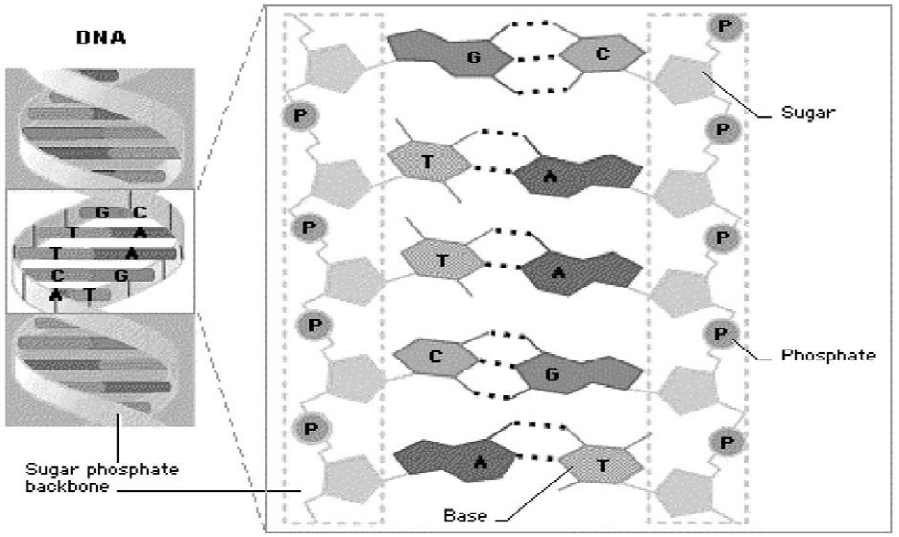
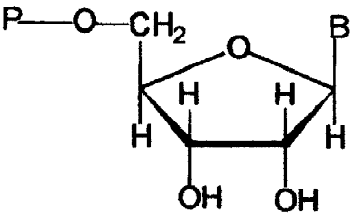


Fig. 1. Three-dimensional structure of DNA showing base pair interactions.



B= Purine or Pyrimidine Base
P= 1,2 or 3 Phosphate Groups

Fig. 2. Nucleotide structure.

these drugs often involve the quantitation and identification of nucleotides or nucleosides. In addition, mutations in the bases and base sequences have been linked to genetic abnormalities, thus nucleic acid constituents may be used as markers for the identification of individuals who are at risk for developing a particular hereditary disease.

Separations of nucleic acids, oligonucleotides, nucleotides, nucleosides and bases have been achieved by two general methods: chromatography and electrophoresis. The chromatographic methods include reversed-phase liquid chromatography (RPLC), ion chromatography (IC) and denaturing HPLC (DHPLC). The electrophoretic methods are slab gel and all modes of capillary electrophoresis (CE). Because of the development of these separations, the way was paved for the start of biotechnology and the field of genomics.

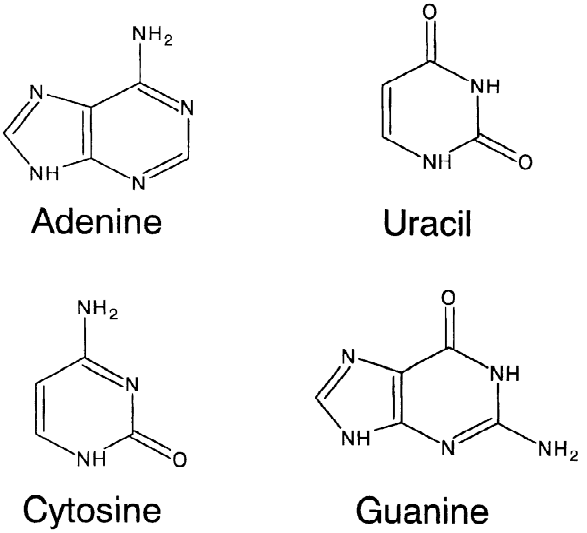


Fig. 3. Structures of purine and pyrimidine bases.

2. HPLC analyses of nucleic acid constituents

2.1. Historical perspectives

Until the middle of the 20th century the analyses of nucleotides were performed by open column adsorption chromatography. In 1949 a publication by Cohn [2] described ion-exchange chromatography. Using this technology nucleotides were separated more quickly and efficiently. In 1967 Horvath et al. [3] reported on the analysis of nucleotides by an instrument called a high-pressure liquid chromatograph. Another article by Horvath and Lipsky [4] followed in 1969 on the analysis of nucleosides and their bases. These analyses were then adapted for use in biological matrices by Brown and co-workers [5–10]. With the development of microparticle, chemically bonded packings, the separations were improved and were applied to studies of the metabolism of nucleic acids and purine and pyrimidine analogs [6]. Fig. 4 shows an early separation of mono-, di- and triphosphates using an anion-

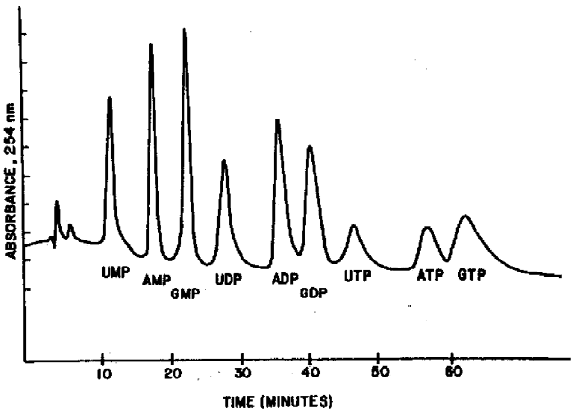


Fig. 4. Separation of mono-, di- and triphosphate nucleotides of adenine, guanine, and uracil. Partisil-10 SAX column, ambient temperature, 0.08 a.u.f.s detector sensitivity. Eluents (low) 0.007 KH_2PO_4, pH 4.0; (high) 0.25M KH_2PO_4, 0.50F KCl, pH 4.5. Linear gradient program rate 1.5 ml/min. From Ref. [6].

exchange column.

Because of significant improvements in column packings, instrument design and the number and kind of detectors commercially available, HPLC became such a vitally important method in the biological and pharmaceutical sciences that today HPLC is used routinely in almost every scientific laboratory that requires good qualitative and quantitative analyses [7–9]. The instrument originally used was a single pump instrument with isocratic elution and a refractive index or single-wavelength UV detector. The columns were 30 cm in length and had an internal diameter of 4.6 mm. They were packed with 37–40 μm pellicular packings [9]. This instrument has evolved into a sophisticated, computerized technique, which can be used for ultra-trace or analytical-scale separations. The present trend in HPLC is in the use of capillary columns. The advantages of these columns are small sample sizes, low eluent consumption and greater ease of interfacing with mass spectrometry (MS). In addition, HPLC has been adapted for preparative or even process-scale separations [8]. At present, a computer is usually integrated with HPLC and CE instruments to control many functions; including separation optimization, control of operating conditions and data analysis.

2.2. Ion-exchange chromatography

Originally, analyses of nucleotides were performed only with anion-exchange chromatography [6]. The retention behavior of nucleotides by ion exchange is predictable with the monophosphate nucleotides being eluted first, followed by the diphosphate nucleotides and then the triphosphate nucleotides [9]. The mechanisms governing the separations are largely electrostatic interactions of the charged analyte with the opposite charge on the stationary phase. However, varying pH, ionic strength and the buffer cation and/or anion of the buffer can change retention time and behavior [7]. In addition, modifiers in the mobile phase as well as temperature of the column affect the retention [7]. The nucleotide separations within each group, i.e., the mono-, di- or triphosphate nucleotides, are dependent on the base. The base order for each group is cytidine, uridine, thymine, adenosine and guanosine. The addition of each phosphate greatly increases the retention time thus the order is all monophosphates, then diphosphates and finally the triphosphates.

In addition, DNA fragments can now be separated by size and nucleotide sequence using non-porous anion-exchange resins [11].

2.3. Reversed-phase liquid chromatography

RPLC is currently the primary mode of separating nucleic acid constituents. Separations are carried out using non-polar columns with alkanes such as C_{18} or C_8 bonded microparticle packings. The separation mechanism of reversed-phase separations is primarily based on the relative polarity of the analytes. However, the retention order of nucleic acid compounds was found to be unpredictable in the original analyses. For example, ribonucleosides, while much more soluble in aqueous solvents than their corresponding bases, have longer retention times [9]. On the basis of solubility and the presence of more hydroxyl groups, it was expected that the ribonucleosides have greater attraction to the polar mobile phase than to the non-polar stationary phases and thus be eluted first. However, a different retention order was found experimentally; the bases were eluted before their corresponding nucleosides. Moreover, although substituent groups on the bases are known to affect retention behavior, the fact that 1-methyladenine was eluted before adenine but 7-methyladenine had an elution time of almost twice that of adenine was difficult to understand [10]. Further investigations revealed a phenomenon known as ‘‘vertical base stacking’’ of the nucleic acid constituents that had a significant effect on the retention time and orders [9].

2.3.1. Vertical base stacking

Base stacking is a vertical association between overlapping purines or pyrimidines. Stacking interactions occur in aqueous solutions and the solid state but do not occur in non-aqueous solvents [10]. The stacking can be either heterogeneous or homogeneous, i.e., between purines or pyrimidines or between a purine and a pyrimidine. Although ribonucleotides, ribonucleosides and their bases have similar stacking patterns despite differences in ionizability and base composition, the extent to which they

stack is different within each group. Stacking only occurs in aromatic structures that contain a nitrogen atom or atoms; thus the presence of nitrogen is required for the stacking process. Most importantly it is postulated that stacking interactions are important stabilizing forces of purine and pyrimidine molecules. Although dipole–dipole moments have little effect on stacking patterns, the stacking appears to result mainly from hydrophobic effects. However, the hydrophobic effects alone cannot account for the stacking process and it is thought that polarization forces play a role. Since non-aqueous solvents complex with aromatic moieties, it is proposed that organic solvents disrupt stacking by associating directly with the purine or pyrimidine bases [10].

Brown and Grushka [10], in their investigation of structure–retention relations in the RPLC of purine and pyrimidine compounds, found that stacking plays a significant role in the retention behavior of nucleosides and their bases. Stacking can help explain the relative order of RPLC retention since the order of retention is the same as the order of effective stacking, i.e., pyrimidines<purines< ribonucleosides<deoxyribonucleosides. Each compound stacks to a different extent. Thus the degree of hydrophobicity of the aggregates is different enough to account for the differences in retention. Compounds where little stacking takes place are the least hydrophobic and have the shortest retention times. Two factors, the existence of a tautomer mainly in the keto form rather than in the enol form and charge formation will cause a decrease both in the extent of stacking and in the retention time. In addition, it was found that any substituent on a purine or pyrimidine ring that can cause charge formation or causes a compound to exist as the keto tautomer rather than the enol tautomer decreases retention (Fig. 5). These groups include OH, NH_2, NHR. The addition of either a methyl group or a ribosyl group to a base more than doubled the retention time and deoxyribonucleosides had longer retention times than their respective ribonucleosides. However, the ribonucleotides had greatly decreased retention times relative to the other compounds. Therefore, the retention behavior may be correlated to ΔG^0 of stacking and/or the equilibrium reaction of $A+A \Leftrightarrow A_2$. Based on this work, Brown and Grushka [10] formulated guidelines that express the relationship between

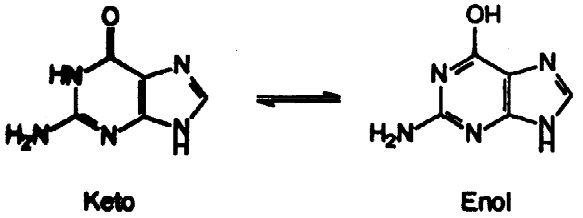


Fig. 5. Lactam–lactim structures. The enol structure contains a hydroxyl group and stacks whereas, the keto structures contain a carbonyl and do not stack.

purine and pyrimidine structures, their ability to stack and their RPLC retention behavior.

Assenza and Brown [12] carried the investigation of structure–retention relationships further by quantifying the relationships for purine compounds in RPLC separations. The quantitative structure–retention relationships (QSRRs) demonstrated the value of functional group contribution terms for both structural elucidation of purines and for the prediction of retention behavior. With these guidelines it is possible to postulate structures from the retention order of unknown purine and pyrimidine derivatives, thus providing some clues to the nucleic acid structure without running more sophisticated analyses such as mass spectrometry or infrared spectroscopy.

An RPLC technique for nucleotides is that of ion pairing [13–17]. Since nucleotides carry negative charges, ion pairing is a useful technique. Both ion-pairing RPLC and anion exchange have been used with nucleotides and other nucleic acid fragments carrying a negative charge.

Finally, DHPLC has also been used for nucleic acid analysis [18–33]. DHPLC is the term given to ion-pairing chromatography of single strands of DNA that are denatured. Partial denaturation is used in studies where two or more chromosomes are compared as a mixture of denatured and reannealed polymerase chain reaction (PCR) amplicons [18]. The homo- and heteroduplex DNA can be distinguished by the respective retentions on RPLC supports. Such conditions have detected single nucleotide substitutions, deletions and insertions [18], thus identifying mutations in genes. Under conditions of full denaturation, polymorphisms have been genotyped. A comparison of DHPLC with direct sequencing for the detection of of single nucleotide poly-

morphisms revealed that for a data set of 41 samples, DHPLC detected all heterozygous sequences found by direct sequencing [19]. DHPLC has been used to study Rett syndrome [20–23], hemophilia A [24], in addition to ovarian [25–30] breast [25–27,30], and colon [31] cancers. DHPLC has also been performed in the capillary format as part of a multiplex system with laser-induced fluorescence (LIF) detection [32].

2.4. Detection

Many detection methods, beside the frequently used UV–visible detection, are now available. They include fluorescence detection [8], mass spectrometry (MS) [33], nuclear magnetic resonance spectroscopy (NMR) [34] and Fourier transform infrared (FT-IR) [35] detection. However, the detection methods most widely used in pharmaceutical companies is MS [33,36–39]. Along with detection of the solutes, the molecular masses and structures can be obtained. Bench-top models of HPLC–MS instruments are now available and the price is considerably lower than that of the original models. The HPLC–MS or HPLC–MS–MS instruments were made possible by the development of the interfaces that eliminated the solvent and made the transfer of the analytes from the HPLC system to the ionization chamber of the MS system feasible. The interfaces commonly used today are the electrospray ionization (ESI) [36,40], matrix-assisted laser desorption ionization (MALDI) [41] and most recently sonic spray ionization (SSI) [42]. Kamel et al. [36] showed that HPLC–ESI-MS was useful in the analysis of the nucleoside antiviral pharmaceuticals; 3′-azido-3′-deoxythymidine (AZT), trifluoruridine, 5-iodo-2′-deoxyuridine and 5′-iodo-2′,3′-dideoxyuridine. The effects of various mobile phase additives, solution pH, and analyte concentration on the electrospray ionization mass spectra of a series of purine and pyrimidine nucleoside antiviral agents were studied in both positive and negative ion modes. It was found that 1% acetic acid gave the greatest sensitivity for $[M+H]^+$ ions and that the use of 50 m*M* NH_4OH gave the greatest sensitivity for $[M+H]^-$ ions. The sensitivities as $[M+H]^+$ were significantly larger than the sensitivities for $[M+H]^-$ ions for the strongly basic purine derivatives. However, the less basic pyrimidine derivatives showed comparable or greater sensitivities as the $[M+H]^-$ ions. In the positive ion mode, the sensitivity as $[M+H]^+$ was independent of the pH of the solution. In the negative ion mode, however, there was an increase in sensitivity with increasing pH and only very weak signals were observed from acidic media. The changes in sensitivity with pH did not match the estimated changes in concentrations of $[M+H]^-$ anions in solution. Abundant adduct ions $[M+NH_4]^+$ for pyrimidines and $[M+OAc]^-$ ions for both the pyrimidines and purines, were formed in ammonium- or acetate-containing solutions. It was suggested that these ions were formed primarily by gas-phase ion–molecule reactions. The relative abundance of the $[M+NH_4]^+$ ions may be related to the gas-phase basicities of the compounds. A method was demonstrated for the analysis of antiviral agents in both negative and positive ion modes by HPLC–ESI-MS–MS (Fig. 6). The method provides effective suppression of adduct formation and could be used for trace analysis of other classes of pharmaceuticals.

Although, HPLC–NMR [34] shows potential for the analysis and structure determination of nucleosides and bases, the problems that still need to be overcome for routine analysis are the high cost of the instrumentation and the need for relatively large size samples. Another technique, which can be used not only to detect the solutes in a sample but also to supply solute identification and structure determination, is HPLC–FT-IR. As long as the eluent can be efficiently removed, solutes can be characterized by their functional groups in the spectra and by spectral matching [35]. This technique is currently being used in our laboratory for the separation and positive identification of the methylated xanthines, caffeine, theobromine and theophylline.

3. Capillary electrophoretic analyses

3.1. Historical perspectives

For many years, slab gel electrophoresis was the separation method most widely used in biological laboratories. Although many samples can be analyzed simultaneously the process is tedious, time consuming and relatively expensive. Therefore, when HPLC was developed, it became the method of

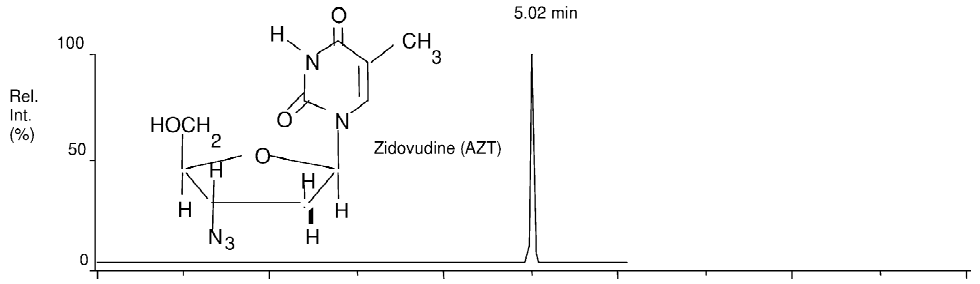


Fig. 6. Representative positive ion ESI-MS (MRM) chromatogram for AZT eluted using a HPLC gradient system of 1% acetic acid (pH 2.8) and methanol with a Luna PLC column (150×4.6 mm, 5 μm ODS). 1 ml/min flow-rate. Gradient: 0–1 min 100% acetic acid, 1–4 min (0–100% methanol), 4–7 min 100% methanol. From Ref. [36].

choice to separate nucleic acids and their building blocks. In the 1980s a new form of electrophoresis became available: CE. This technique is performed in fused-silica capillaries and has advantages over slab gel electrophoresis in terms of speed, cost, resolution and automation [8]. A direct clinical comparison of slab gel electrophoresis and CE for the detection of short tandem repeat fragments of DNA found similar sizing capabilities were obtained with automated CE as were found with conventional slab gel electrophoresis [43].

CE has several advantages in comparison to HPLC. CE is less expensive and faster. In addition, it has higher efficiency and can handle much smaller sample volumes. The smaller sample volume makes it particularly suitable for clinical and forensic separations [44].

3.2. Modes of separation

CE has many modes; capillary gel electrophoresis (CGE), capillary electrochromatography (CEC), capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) [8]. Each mode can be used for a different purpose. For example, in CGC the capillary is filled with a gel and the molecules are separated on the basis of size. CEC combines the advantages of CE with those of HPLC. In CEC, the capillary is either filled with packing or the walls are coated with a stationary phase; thus separations are obtained based on partitioning of the solutes between a stationary and a mobile phase as well as by electrophoretic mobility. By the addition of micelles to the buffer, neutral molecules as well as charged moieties can be separated by MEKC. Thus there are many CE mechanisms that are available for the separation of solutes by size, shape or charge.

CE has been used for the analysis of nucleic acids, oligonucleotides and the smaller building blocks of DNA; nucleotides, nucleosides and bases. An extensive applications review was published by Righetti and Gelfi on the subject of CZE techniques for the analysis of diagnostic DNA products [45].

3.3. Separation optimization

Because of the small sample volume and thus the min amount of analyte; sample preconcentration techniques have been applied to CE. Whole capillary stacking [46] and isotachophoresis [47] have been performed in-situ to preconcentrate sample constituents and enhance detection sensitivity. Whole capillary stacking was performed on the anti-human immunodeficiency virus metabolite carbovir triphosphate [48]. Stacking lowered the detection limit from 4 to 0.02 μ*M*. The electropherogram of the separation of carbovir triphosphate from the triphosphate deoxyribonucleosides of adenine and guanine is shown in Fig. 7.

Optimal separation conditions for CE using bare fused-silica capillaries for nucleotide separations were investigated [49–51]. High pH values for buffers (30–50 m*M*) were best for ribonucleotide separations [51], as the pH of the buffer was well above the pK_a values of the nucleic acid constituents, thus stabilizing the charge. The separation of all 12 nucleotides with the carbonate buffer system at pH 10 is shown in Fig. 8. In further studies of the effects

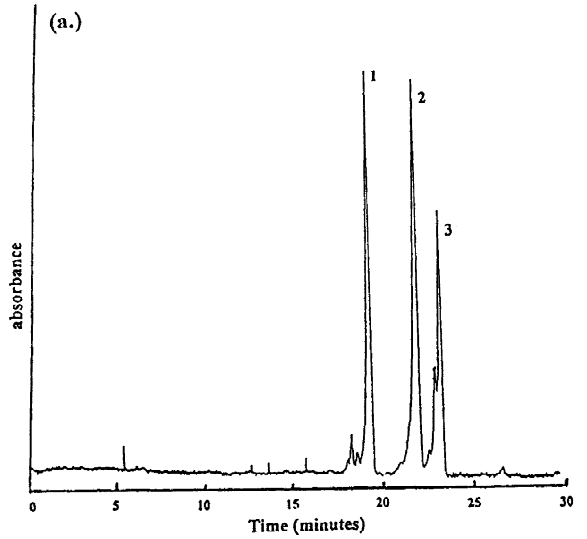


Fig. 7. CE separation of carbovir triphosphate from ATP and GTP with whole capillary stacking. From Ref. [48].

of different buffer anions and cations, carbonate was the most effective anion and potassium the best cation for nucleotide separations [52]. However, since volatile buffers are necessary with MS and FT-IR detectors, ammonium carbonate was investigated as part of these studies and gave adequate separations [52]. In a separate study, oligonucleotide separations were optimized at low pH in contrast to the high-pH separations. Single base differences of the oligonucleotides could be detected by the method used [53].

The adsorption of analytes onto the capillary surface can be reduced by the application of a coating to the fused-silica surface [54]. Surface modified fused-silica capillaries have also been used to attain nucleotide separations. The Ucon coating developed and tested by Zhao et al. [55] separated all 12 ribonucleotides in less than 20 min. Fig. 9 shows the separation obtained with the coated capillary.

A double-strand polyaniline-based polymer is currently under investigation in our laboratory and is useful in enhancing nucleotide and nucleobase separations. A unique characteristic is that the separation is tunable by pH. Above a pH of 6, the solution is hydrophobic and below it is hydrophilic. In addition, depending on the oxidation state of the polyaniline, the polymer can be electroactive or insulating [56].

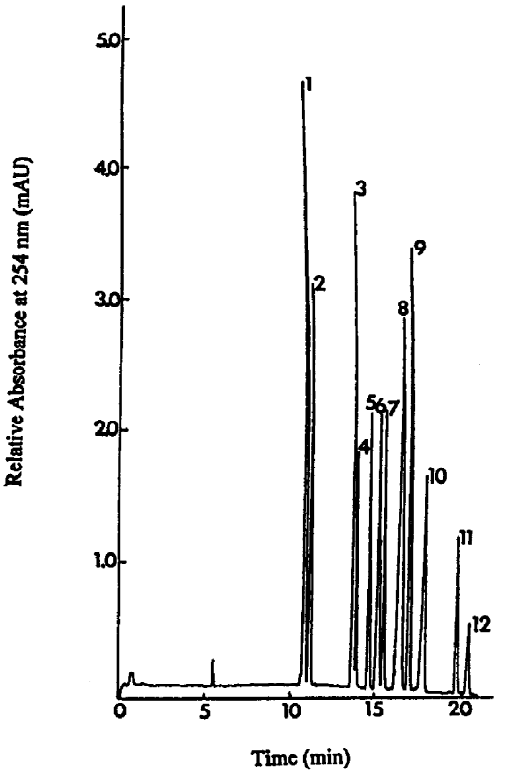


Fig. 8. CE separation of 12 ribonucleotides (~0.01 m*M*). Conditions: 10 s hydrodynamic injection (10 cm); capillary 70 cm (60 cm separation distance)×75 μm I.D. column; buffer: 30 m*M* sodium carbonate/hydrogencarbonate, pH 10; voltage +18 kV; detection: 254 nm. Peaks: 1=AMP, 2=CMP, 3=ADP, 4=GMP, 5=CDP, 6=ATP, 7=UMP, 8=CTP, 9=GDP, 10=GTP, 11=UDP, 12=UTP. From Ref. [51].

In addition to surface coatings, CEC has been used for nucleoside separations. In this mode, the capillary is packed with a stationary phase but an electric field remains the driving force for the separation [57,58].

3.4. Detection

Nucleic acid constituents separated by CE are most commonly detected by UV. However, both MS and fluorescence detection are increasingly popular. The ionization methods used most frequently with CE–MS are fast atom bombardment (FAB) and ESI [59]. CE–MS has been used for the analysis of nucleotides and nucleosides [60]. However, the technique is not being used routinely, although MS shows promise for use with hybridization techniques

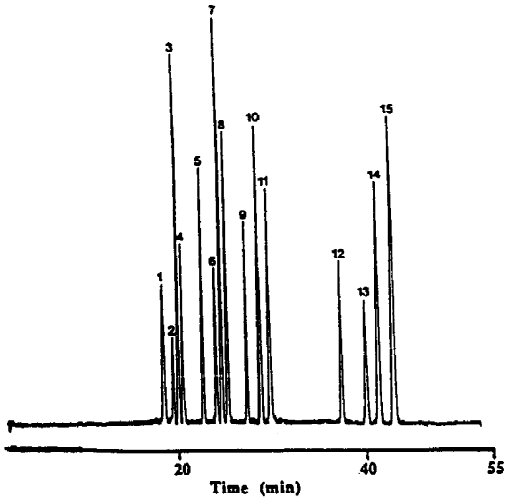


Fig. 9. CE separation of ribonucleotide standards on a Uconcoated column. Conditions: 90 cm (54 cm separation distance)× 50 μm I.D. column; 30:50 m*M* phosphate:Tris–HCl buffer, pH 5.2; electromigration injection for 6 s at −23 kV; −20 kV applied voltage. Peaks: 1=UTP, 2=CTP, 3=ATP, 4=GTP, 5=UDP, 6=CDP, 7=ADP, 8=GDP, 9=XMP, 10=UDP-g, 11=ADP-r, 12=UMP, 13=CMP, 14=AMP, 15=GMP. From Ref. [55].

[61]. The major limitation of CE–MS is poor concentration sensitivity [59]. Although sample preconcentration techniques can enhance sensitivity [46,47], the concentration detection limits of the techniques remain relatively high relative to HPLC methods [59]. Recent advances in CE–MS have been highlighted in the review by von Brocke et al. [62].

LIF detection, which has been coupled to CE [44] gives excellent sensitivity (up to 10^{-12} *M*) and can detect several analytes in one run. Although relatively few analytes are naturally fluorescent, the analytes can be derivatized prior to analysis with a fluorescence label [63]. LIF detection is extremely well suited to the analysis of nucleic acid constituents and is the standard detection method for DNA sequencing [61]. The ability to selectively label constituents provides a very sensitive analytical tool for the analysis of nucleic acid constituents of many sizes. With CE–LIF nucleic acid constituents of many sizes can be determined. Specific nucleotides such as adenine [64], single nucleotide polymorphisms [61] have been determined in DNA samples in addition to DNA reassociation studies [65].

4. Future developments

Future enhancements of CE in the field of genomics include the development of more fluorescent labels [61] and technologies that will increase the speed of the analyses. Integrated systems, which are capable of performing DNA amplification fluorescent labeling, and separation and identification of the base sequence have already been developed [66,67]. The ability to obtain multiple separations in several capillaries at the same time has been made possible by the use of multiple capillary array instruments. A typical array instrument holds about 100 capillaries, however, an instrument developed by Dovichi [68] can hold 576 capillaries. The detectors in array systems have been redesigned to integrate with the capillary arrays [69–71]. For both HPLC and CE, chip based separations are now available and will be an important asset in the future [72]. Although, data analysis systems are being adapted to cope with the vast data sets that are being obtained [73,74]. The remaining problem is ‘‘throughput’’ driven; how to analyze more samples in less time and develop data systems capable of keeping up with the data produced.

5. Conclusion

HPLC and CE have played a significant role in biotechnology and the field of genomics. Looking back on the late 1960s, the deciphering any genome and especially the human genome looked like an almost impossible task that would take decades to accomplish. The development of reliable, efficient separation technologies such as HPLC and CE was the foundation on which the first steps could be taken in this quest. The refinement of these technologies and the production of miniaturized, automated and hyphenated instruments accelerated the research to an unimaginable degree. The problems encountered were solved and giant steps were taken to find the answers of the human genome code. One of the great advances in the past two decades has been the integration of scientists, with biologists, chemists and physicists all working together to attack a vast scientific problem. We learned to communicate with each other and we found out what we could do with

combined resources and ideas. The next decades will be exciting! There will be new advances in genetics and medicine with new therapies available and we can look forward to a new era of long life with good health.

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