Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1076 (2005) 83–89
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Effects of sequence and structure in the separation of nucleic acids using ion pair reverse phase liquid chromatography

Mark J. Dickman *

Biological and Environmental Systems Group, Department of Chemical and Process Engineering, University of Sheffield, Mappin Street, Sheffield S1 3JD, UK

Received 4 January 2005; received in revised form 1 March 2005; accepted 4 April 2005

Abstract

Ion pair reverse phase high performance liquid chromatography on non-porous alkylated poly(styrene-divinylbenzene) particles enables the high resolution separation of double stranded DNA fragments. To further understand the separation mechanisms involved in ion pair reverse phase liquid chromatography we have analysed the effects of curved or "bent" DNA fragments with respect to their separation using both gel electrophoresis and ion pair reverse phase liquid chromatography. Size dependent separations of curved DNA fragments that migrate anomalously during gel electrophoresis were observed using ion pair reverse phase liquid chromatography. To further study the sequence effect and resulting changes in hydrophobicity of the duplex DNA, PCR fragments were generated that contain uracil in place of thymine. The resulting fragments were shown to elute with shorter retention times, demonstrating that sequence-specific effects can alter the retention of duplex DNA. The study was extended to the investigation of non-canonical B-DNA structures (Holliday junctions) under various chromatographic conditions, demonstrating that the coaxial stacking of the helices in such structures, in the presence of magnesium causes a change in retention.

Keywords: Ion pair reverse phase liquid chromatography; Nucleic acids; Non-porous alkylated poly(styrene-divinylbenzene) particles

1. Introduction

Ion pair reverse phase high performance liquid chromatography (IP RP HPLC) offers significant advantages and opportunities for the analysis of nucleic acids. The non-porous polymeric medium, in conjunction with the highly monodisperse nature of the particles, results in the minimisation of the diffusion paths (reviewed in [1]) and therefore provides high resolution with very short analysis times. Furthermore, the column media is robust, being resistant to a broad range of temperatures and pH. This has led to a wide variety of developments in the analysis of nucleic acids using IP RP HPLC [2], including the sequence independent sizing of duplex DNA (up to 2000 base pairs (bps)) under non-denaturing conditions [3]. Chromatography under denaturing conditions facilitates the analysis of oligonucleotides [4], the separation of RNA [5–7] and the separation and purification of single-stranded (ss) DNA [8]. Using partially denaturing conditions heteroduplex analysis can be performed, enabling the detection of single mismatches in DNA fragments (reviewed in [9]). IP RP HPLC also provides a versatile platform for the rapid analysis of nucleic acid modification reactions [10]. Further studies have also demonstrated the ability of IP RP HPLC to indirectly study RNA conformation and DNA-protein interactions by analysing the products of DNA and RNA footprinting reactions [7,11].

Here it is demonstrated that IP RP HPLC allows the size dependent separation of curved or bent duplex DNA with no significant effect of the end to end length of the DNA on chromatographic retention. The effects of nucleotide sequence and the consequent changes in hydrophobicity of the duplex DNA are investigated by incorporating uracil into the DNA fragments in place of thymine.

* Tel.: +44 114 222 7541; fax: +44 114 222 7566.
E-mail address: m.dickman@sheffield.ac.uk.

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.04.018

The results demonstrate that sequence dependent effects can influence the retention of duplex DNA. The separation and analysis of non-uniform B-DNA structures were also investigated with the analysis of synthetic Holliday junctions under various chromatographic conditions. The results show that large non-canonical B-DNA structures no longer separate in a size dependent fashion. Furthermore, it was observed that through the incorporation of magnesium ions in the chromatography, the synthetic Holliday junctions appeared to adopt a more compact structure, through coaxial helical stacking, resulting in increased retention following chromatography.

2. Materials and methods

2.1. HPLC analysis

All samples were analysed by IP RP HPLC on an Agilent 1100 HPLC (Agilent Palo Alto) using a DNAsep column 4.6 mm × 50 mm i.d. (Transgenomic, San Jose). The stationary phase of the column consists of a non-porous, alkylated poly(styrene-divinylbenzene) matrix. Chromatograms, analysed using UV detection, were recorded at a wavelength of 260 nm.

2.2. IP RP HPLC

The IP RP HPLC analysis was performed using the following conditions: buffer A 0.1 M triethylammonium acetate (TEAA) (Fluka), pH 7.0; buffer B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile. The pBR322 *Hin*fI/*Eco*RV/*Ssp*I triple restriction digest and a *Hae*III digest of pUC18 (Bioline) was analysed using the following gradient [1]: starting at 35% buffer B the gradient was extended to 50% buffer B in 3 min, followed by an extension to 65% buffer B over 15 min at a flow rate of 1.0 ml/min. Ninety-six base pairs PCR fragments were analysed using the following gradient [2]: Starting at 25% buffer B the gradient was extended to 35% buffer B in 3 min, followed by an extension to 55% buffer B over 15 min at a flow rate of 1.0 ml/min. The 255 bp and 440 bp PCR fragments were analysed using gradient 1.

The IP RP HPLC analysis of the synthetic Holliday junctions was performed using gradient 1. With the incorporation of 1 mM $MgCl_2$ into the chromatography buffers the analysis was performed using the following gradient [3]. Starting at 20% buffer B the gradient was extended to 35% buffer B in 3 min, followed by an extension to 50% buffer B over 15 min at a flow rate of 1.0 ml/min.

2.3. Synthesis and purification of oligodeoxynucleotides used in the binding studies

Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA synthesiser using cyanoethyl phosphoramidite chemistry.

Oligodeoxynucleotides were provided in solution after deprotection in 30% ammonia. The oligonucleotides were purified using denaturing PAGE and subsequently evaporated to dryness and desalted using a Pharmacia NAP 10 column according to the manufacturer's instructions. Synthetic Holliday junctions, HJ50 and HJ80, each comprised four 50-mer and four 80-mer oligonucleotides. DNA annealing and purification were essentially as described previously [12]. HJ50 contained HJ1, 2, 3 and 4. HJ1 (5′-CCTCGAGGGATCCGTCCTAGCAAGCCGCTGCTACC-GGAAGCTTCTCGA GG-3′). HJ2 (5′-CCTCGAGAAGCT-TCCGGTAGCAGCGAGAGCGGTG GTTGAATTCCTC-GAGG-3′). HJ3 (5′-CCTCGAGGAATTCAACCACCGCT-CT TCTCAACTGCAGTCTAGACTCGAGG-3′). HJ4 (5′-CCTCGAGTCT AGACTGCAGTTGAGAGCTTGCTAGG-ACGGATCCCTCGAGG-3′) HJ80 contained HJ5, 6, 7 and 8. HJ5 (5′-CGCAAGCGACAGGAACCTCGA GGGATCCG-TCCTAGCAAGCCGCTGCTACCGGAAGCTTCTCGAGG TTCCTGTCGCTTGCG-3′). HJ6 (5′-CGCAAGCGACA-GGAACCTCGAGAA GCTTCCGGTAGCAGCGAGAGC-GGTGGTTGAATTCCTCGAGGTTCCTGTC GCTTGCG-3′). HJ7 (5′-GGCAAGCGACAGGAACCTCGAGGAATT-CAACCA CCGCTCTTCTCAACTGCAGTCTAGACTCG-AGGTTCCTGTCGCTTGCG-3′). HJ8 (5′-CGCAAGCGA-CAGGAACCTCGAGTCTAGACTGCAGTTGAGAGCTT GCTAGGACGGATCCCTCGAGGTTCCTGTCGCTTGC-G-3′).

2.4. Restriction digestion of pBR322

Five micrograms of pBR322 was incubated with 10 U *Hin*fI (Fermentas), 10 U *Eco*RV (Promega) and 15 U *Ssp*1 (Fermentas) in a total volume of 50 μl at 37 °C overnight. Following digestion the products were analysed using IP RP HPLC and polyacrylamide gel electrophoresis.

2.5. Polyacrylamide gel electrophoresis

Five to ten percent non-denaturing polyacrylamide gels were prepared using acrylamide:bisacrylamide 29:1 (Bio-Rad) buffered with 45 mM Tris–borate (pH 8.0), 1 mM EDTA and run for 1 h, 100 V at 25 °C. PAGE gels containing magnesium were buffered with 45 mM Tris–borate (pH 8.0), 1 mM $MgCl_2$ and run for 1 h, 100 V at 25 °C. The gels were visualised after staining with fluorescent dyes SYBR green for dsDNA (Sigma) using a Transilluminator.

2.6. PCR

Fifty microlitres PCR reactions contained 20 mM Tris–HCl pH 8.0, 63 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (either dA, dG, dC, dTTP or dA, dG, dC and dUTP) 0.2 μM primer 1 (5′-ACAGCATCGCCAGTCACTAT-3′), with either 0.2 μM primer 2 (5′-CAAAGCGGTCGGACAGTG-CT-3′) to generate the 96 bp product, or 3 (5′-TGATGTC-GGCGATATAGGCG-3′) to generate the 255 bp product

or 0.2 μM primer 4 (5′-ATGCGACTCCTGCATTAGGA-3′) to generate the 440 bp product, 5 ng of pBR322 and 2 U taq polymerase (Bioline, UK). The reaction mixture was then incubated for 2 min at 95 °C prior to PCR. The PCR conditions used were 95 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s for 30 cycles. At the end of the amplification phase a 5 μl fraction of the PCR product was directly injected onto the DNASep column.

3. Results and discussion

3.1. Analysis of curved DNA using IP RP HPLC

Fragments of DNA with reduced electrophoretic mobility are commonly regarded as curved and are universally present at or near the site of replication, transcription factor binding or recombination. Poly A tracts have been identified as the major characteristic of curved regions of DNA (reviewed in [13]), that have been identified through a variety of structural studies. In addition curved DNA fragments have also been identified using electric birefringence [14], hydroxyl radical footprinitng [15] and electron microscopy [16]. Curved DNA fragments have been shown to possess reduced mobility during gel electrophoresis [17–19], as migration is dependent on end to end length [20]. Curved DNA fragments also show retardation during anion exchange HPLC, through the preferential attachment of the curved DNA to the ionic groups of the column [21]. The effect of curved DNA in IP RP HPLC was examined using the restriction digestion of pBR322 with *Hin*fI, *Ssp*I and *Eco*RV. It has been shown previously that the tn3 region of pBR322 (between *Ssp*I *Hin*dIII site contains an intrinsic bend [22]) this corresponds to the 378 bp fragment generated during the digest. The restriction fragments were

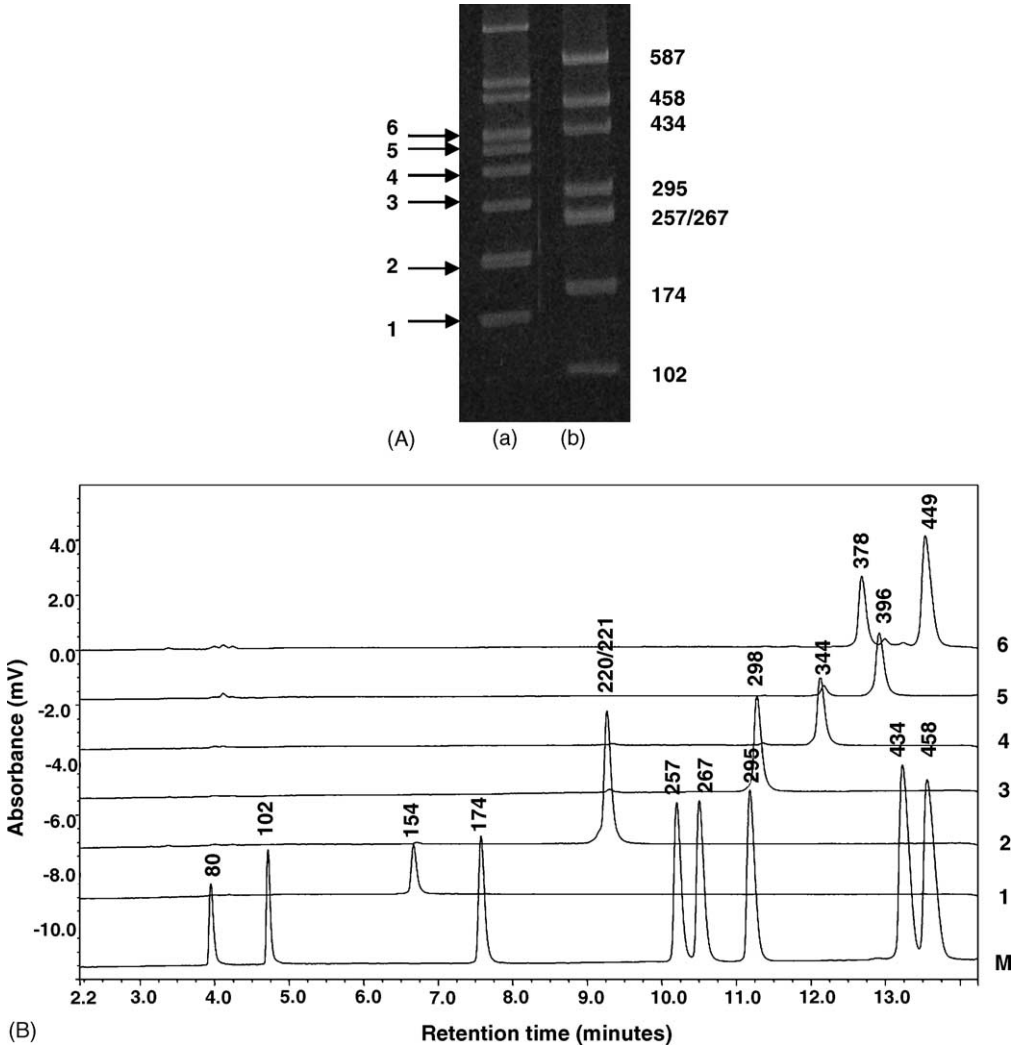

Fig. 1. Analysis of the pBR322 *Hin*fI, *Eco*RV, *Ssp*I digest using gel electrophoresis (A) and IP RP HPLC (B). (A) Lane (a) contains the pBR322 *Hin*fI, *Eco*RV, *Ssp*I digest and lane (b) pUC18 *Hae*III digest (the size of the bands in base pairs are highlighted). Bands 1–6 were eluted from the gel and analysed using IP RP HPLC as shown in (B). (B) IP RP HPLC analysis. Trace M contains the pUC18 *Hae*III digest lanes 1–6 contain fragments eluted from the PAGE, all sizes of the DNA are indicated.

analysed using both IP RP HPLC and gel electrophoresis, individual bands from the polyacrylamide gel were eluted and subsequently analysed using IP RP HPLC (see Fig. 1). The results show the size dependent chromatography for the elution of the bands 1–5 (fragments 154, 220/221, 298, 344 and 396) eluted from the polyacryalmide gel when analysed using IP RP HPLC, in comparison to the size standards generated from a pUC18 *Hae*III digest. However, the analysis of band 6 from the polyacrylamide gel shows that two species are observed during IP RP HPLC. The results show that the 378 bp fragment co-migrates with the 447 bp fragment during gel electrophoresis. The 378 bp fragment has previously

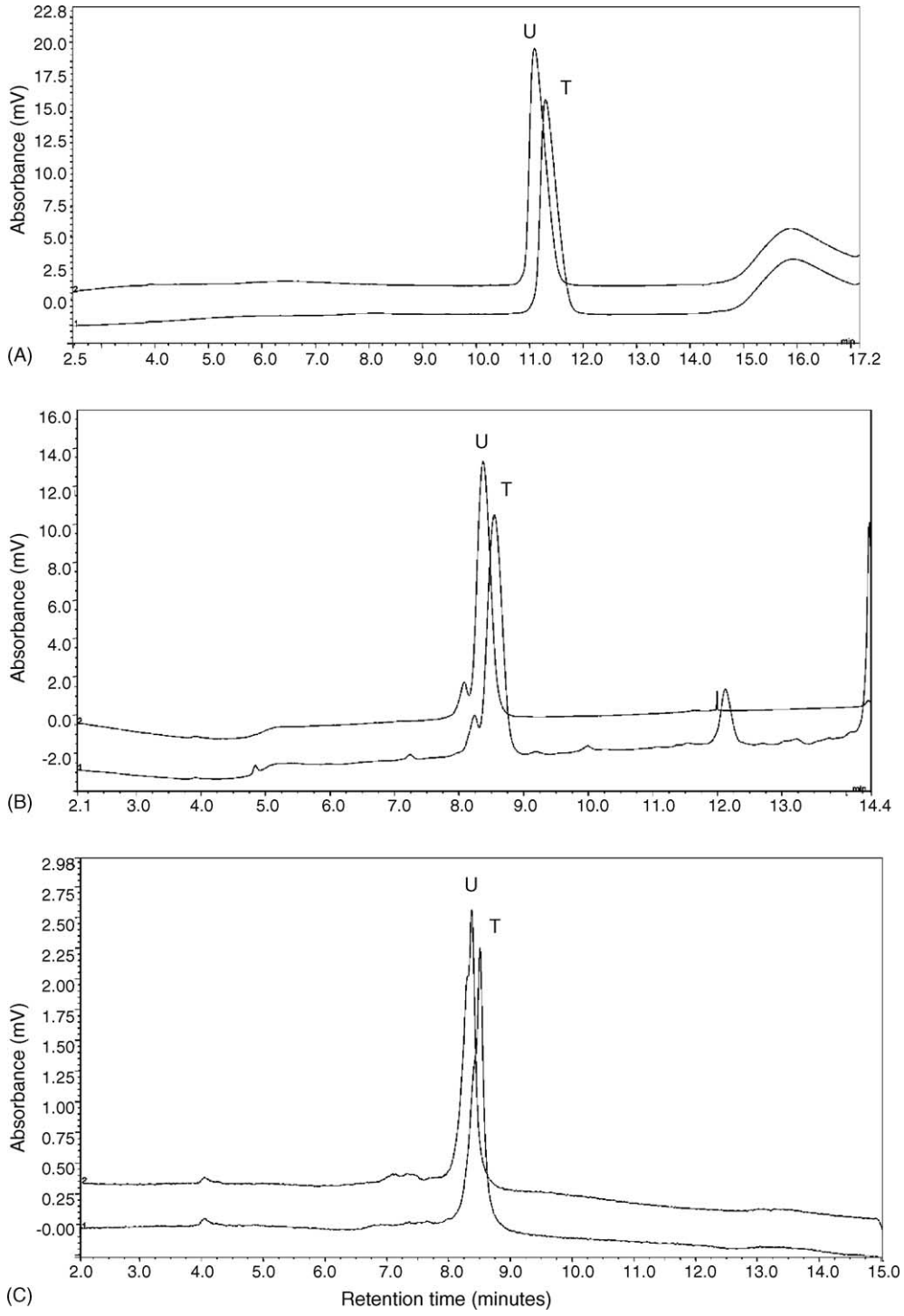

Fig. 2. IP RP HPLC analysis of duplex DNA containing uracil. PCR fragments were generated using dUTP to create DNA duplexes that contained uracil instead of thymine (see Section 2). The IP RP HPLC analysis of both the uracil containing (U) and thymine containing (T) DNA products are shown. (A) Shows the 440 bp, (B) the 255 bp and (C) the 96 bp PCR products.

been identified as an intrinsically bent DNA fragment, which runs with retarded migration during electrophoresis and retarded elution during ion exchange HPLC [17]. However, under IP RP HPLC conditions there is no anomalous retention of the 378 fragment (see Fig. 1B), demonstrating that the end to end length of a DNA molecule has no significant effect on the retention time. These data further demonstrate the use of IP RP HPLC as an accurate method for the sizing of DNA fragments with the curvature of the DNA resulting in no significant alteration of the retention of the DNA.

3.2. Sequence effects in the separation of duplex DNA

It has previously been demonstrated that the separation of duplex DNA using IP RP HPLC is essentially size dependent [3] with little or no effects sequence or structure in the retention of DNA (as observed with the pBR322 *Hin*fI, *Eco*RV, *Ssp*I digest). The separation of ssDNA is sequence dependent using IP RP HPLC where hydrophobicity of the homooligonucleotides increases in the order C < A < T. No influence of AT content was observed in duplex DNA retention using IP RP HPLC [3] although it is known that AT base pair rich sequences are capable of preferentially binding to triethylammonium ions and therefore would be expected to be retained on the columns longer than GC rich sequences. Since the elution behaviour of duplex DNA does not reveal any dependence on AT content it was assumed that the separation of duplex DNA is achieved through a combination of both ion pairing and dynamic ion exchange [3]. However, perturbations to entirely size based separations of DNA using IP RP HPLC do exist. It has previously been demonstrated that fragments of identical size do not always co-elute and unpredictable effects on retention time are observed [3]. These differences are not readily explained in terms of base sequence. To further investigate the effects of base sequence a range of PCR products (96, 255 and 440 bp) were generated that contained uracil instead of thymine by the incorporation of dUTP during the PCR. The hydrophobicity of uracil is lower than thymine (through the loss of the C5 methyl group) and has a shorter retention time under reverse phase conditions [23]. IP RP HPLC analysis of the generated fragments is shown in Fig. 2. From the results it can be seen that the duplex DNA containing uracil elutes before the thymine containing duplex DNA. These results were repeated over a range of temperatures from 35 to 50 °C (data not shown), with similar differences in retention between the DNA duplexes, demonstrating that partial denaturation does not account for the difference in retention time of the duplex DNA. These results show that the presence of uracil results in the earlier elution of the DNA fragment through a reduction in the overall hydrophobicty of the DNA duplex, demonstrating that sequence-specific effects, similar to those observed in the separation in ssDNA, can affect the retention of duplex DNA fragments.

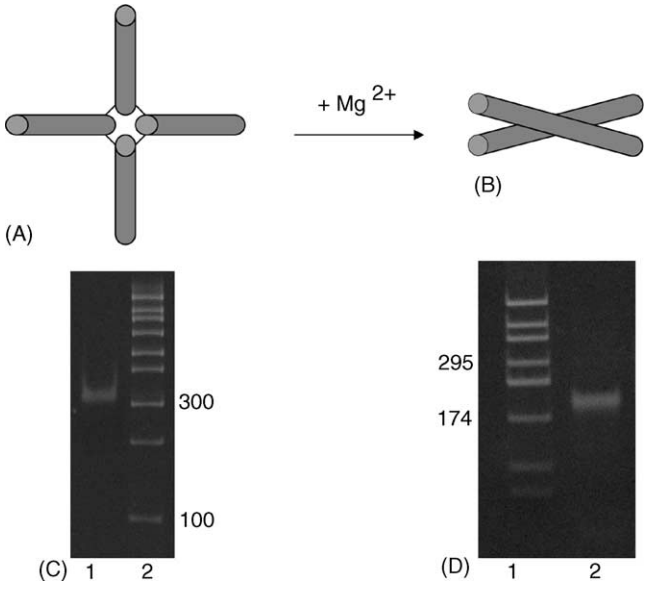

Fig. 3. Synthetic Holliday junction conformations. (A) Shows a schematic representation of the extended four-fold symmetrical structure of the Holliday junction. (B) Shows a schematic representation of the stacked X structure of the Holliday junction that involves the pairwise coaxial stacking of the helical arms. (C) Shows the PAGE analysis of the Holliday junction (HJ50) in the absence of magnesium (lane 1). Lane 2 contains a 100 bp ladder (Bioline). (D) Shows the PAGE analysis of the Holliday junction (HJ50) in the presence of magnesium (lane 2). Lane 1 contains a pUC18 *Hae*III digest.

3.3. Analysis of non-canonical B-DNA structures using IP RP HPLC

The Holliday junction is a branched form of DNA comprising of four DNA helices connected together by the covalent continuity of the four component strands (see Fig. 3A). The gel electrophoretic properties of stable junctions in linear DNA molecules showed that Holliday junctions have the effect of bending or kinking the DNA at that point [24]. The fragments migrate anomalously slow in native polyacrylamide gels, and the extent to this anomaly depends on the relative position of the junction relative to the ends of the fragment in a similar fashion to curved DNA fragments [19]. The mobility of the Holliday junctions is also dependent on cation concentration; magnesium was shown to be very effective in inducing a compact conformation [19].

The complete DNA junctions migrate as a narrow band consistent with the formation of a structurally well defined species (see Fig. 3C and D). The synthetic Holliday junction migrates anomalously slow in native polyacrylamide gels (see Fig. 3C) which shows that the 100 bp Holliday junction migrates above the 300 bp duplex DNA marker. In the presence of Mg^{2+} ions the mobility of the Holliday junction is altered (see Fig. 3D). From the gel it can be seen that the it now runs with a faster mobility (approximately 200 bp) compared to the absence of cations, indicating a more compact conformation as shown in Fig. 3B.

The chromatographic analysis of the Holliday junction under non-denaturing conditions (40 °C) is shown in Fig. 4.

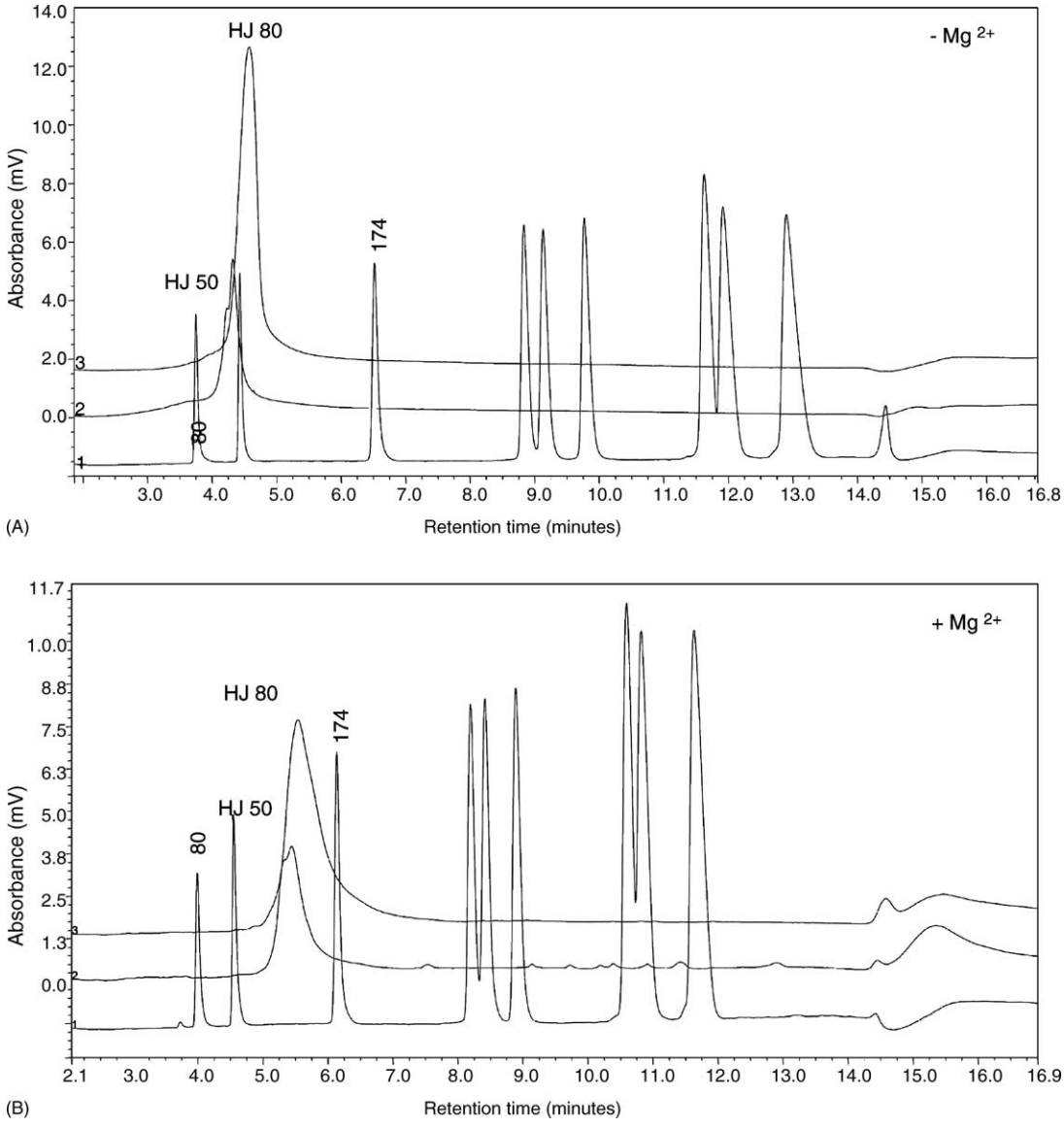

Fig. 4. IP RP HPLC analysis of the synthetic Holliday junctions. Figure shows the IP RP HPLC analysis of the Holliday junctions HJ50 and HJ80 in both the absence (A) and presence of magnesium incorporated into the running buffers (B). The pUC18 *Hae*III digest is shown with the 80 and 174 bp fragments highlighted.

The smaller Holliday junction (HJ50) runs with a retention time corresponding to 100 bp duplex fragment (4×25 bp arms), no anomalous migration is seen compared to gel electrophoresis, consistent with the results demonstrating that curved DNA has no significant effect on retention time. However, the results also show that the elution of the larger Holliday junction (HJ80) corresponding to a similar sized DNA species. The larger HJ80 is expected to elute at approximately the same time as a 160 bp duplex fragment (4×40 bp arms). These results also demonstrate that the larger Holliday junctions appears as broader peak (possibly heterogeneous species).

Fig. 4B shows the IP RP HPLC analysis of the synthetic Holliday junctions in the presence of 1 mM Mg^{2+} in the running buffers. The magnesium causes the duplex DNA fragments to elute with altered retention times, to optimise the resolution of the duplex DNA fragments an alternative gradient was used to that previously (see Section 2) therefore the specific retention times in Fig. 4A and B are not comparable. However, the retention in comparison to the duplex DNA can be made. From the results it can be seen that the presence of the magnesium cations causes the relative retention of both HJ50 and HJ80 to increase when compared to the retention of duplex DNA. From the chromatogram it can be seen that the presence of magnesium the synthetic Holliday junctions are eluted nearer to the 174 bp duplex DNA fragment (see Fig. 4B) when compared to the elution in the absence of magnesium ions (see Fig. 4A). Therefore the stacked conformation of the Holliday junction results in the later elution compared to the retention time of corresponding duplex DNA

in the absence of magnesium. The magnesium induced conformation under electrophoretic conditions migrates more quickly due to a decrease in the end to end distance (a more compact structure, see Fig. 3B). Previous results have shown that the end to end length of DNA fragments does not significantly effect the retention time under IP RP HPLC conditions. Therefore, it is proposed that the increase in retention results from an increase in hydrophobicity of the stacked DNA species, the coaxial helical stacking (induced by magnesium ions) confers additional hydrophobicity of the DNA species through an increase in the contact area of the synthetic Holliday junction available for interaction with the hydrophobic surface of the column. This is a similar mechanism to the separation of partially denatured DNA which contains ss-DNA regions and therefore in comparison to entirely double stranded helical DNA are smaller and subsequently have a smaller contact are that is able to interact with the hydrophobic column [1].

4. Conclusions

IP RP HPLC enables the size dependent separation of duplex DNA, no significant effects of end to end length or curvature of DNA on retention time of duplex DNA fragments is observed. This is in contrast to anion exchange, capillary electrophoresis and slab gel electrophoresis. However, small effects in base composition and hydrophobicity of the duplex DNA is shown to effect the retention time and influence the separation of duplex DNA. The separation of large non-canonical B-DNA structures such as the Holliday junction are not entirely resolved in a size dependent mechanism using IP RP HPLC.

Moreover, the folding of such structures in the presence of divalent cations causes a change in retention of the Holiday junction, with the more compact junctions (through stacking of the helicies) causing an increase in retention time when compared to duplex DNA under the same conditions. The results indicate that ion pair reverse liquid chromatography may be a useful tool in examining the folding of complex RNA and DNA structures to analyse the helical stacking and factors affecting stabilisation of such structures.

Acknowledgement

The author would like to thank David Hornby for his helpful comments and discussions regarding the manuscript.

References

[1] C.G. Huber, J. Chromatogr. A. 806 (1998) 3.
[2] D.T. Gjerde, C.P. Hanna, D.P. Hornby, DNA Chromatography, Wiley-VCH, Weinheim, 2002.
[3] C.G. Huber, P.J. Oefner, G.K. Bonn, Anal. Chem. 67 (1995) 578.
[4] C.G. Huber, P.J. Oefner, G.K. Bonn, Anal. Biochem. 212 (1993) 351.
[5] D.E. Georgopoulos, M.J. Leibowitz, J. Chromatogr. A 868 (1999) 109.
[6] A. Azarini, K.H. Hecker, Nucleic Acids Res. 29 (2001) 2.
[7] M.J. Dickman, M.J. Conroy, J.A. Grasby, D.P. Hornby, RNA 8 (2002) 247.
[8] M.J. Dickman, D.P. Hornby, Anal. Biochem. 284 (2000) 64.
[9] W. Xiao, P.J. Oefner, Hum. Mutat. 6 (2001) 439.
[10] M.J. Dickman, M.M. Matin, D.P. Hornby, Anal. Biochem. 301 (2002) 290.
[11] M.J. Dickman, S.E. Sedelnikova, J.B. Rafferty, D.P. Hornby, J. Biochem. Biophys. Methods 58 (2004) 39.
[12] C.A. Parsons, I. Tsaneva, R.G. Lloyd, S.C. West, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5452.
[13] P.J. Hagermann, Annu. Rev. Biochem. 59 (1990) 755.
[14] P.J. Hagermann, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 4632.
[15] A.M. Burkhoff, T.D. Tullius, Cell 48 (1987) 935.
[16] J. Griffith, M. Bleyman, C.A. Rauch, P.A. Kitchin, P.T. Englund, Cell 46 (1986) 717.
[17] J.C. Marini, S.D. Levene, D.M. Crothers, P.T. Englund, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 7664.
[18] N.C. Stellwagen, Biochemistry 26 (1983) 6186.
[19] H.M. Wu, D.M. Crothers, Nature 308 (1984) 509.
[20] O.J. Lumpkin, P. Dejardin, B.H. Zimm, Biopolymers 8 (1985) 1573.
[21] F. Fack, V. Sarantoglou, Nucleic Acids Res. 15 (1991) 4181.
[22] P. Prentki, M.H. Pham, D.J. Galas, Nucleic Acids Res. 15 (1987) 10060.
[23] C.W. Gehrke, J. Chromatogr. 301 (1984) 199.
[24] G.W. Gough, D.M. Lilley, Nature 313 (1985) 154.