Ion Pair Reverse-Phase Chromatography: A Versatile Platform for the Analysis of RNA

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Introduction

The requirement for high throughput analytical tools that can readily separate, purify and analyse ribonucleic acids (RNA) are assuming increasing significance with the recent discoveries of the diverse and important roles RNA plays in biological systems. RNA consists of a long chain of nucleotide units made up of a nitrogenous base, a ribose sugar, and a phosphate. Cells contain three abundant classes of RNA including messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), these can be classified as RNAs involved in protein synthesis. In addition to these abundant RNAs involved in protein synthesis a wide range of cellular RNAs exist within the cell. Our appreciation of the relative importance of RNA in numerous biological processes has increased substantially over recent years. The discoveries of catalytic RNA [1-2], RNA interference and non-coding regulatory RNAs [reviewed in 3-4], and the recent association with mediating antiviral response in prokaryotes [5-6] have all impacted a broad range of disciplines. In addition, the utilisation of RNA as a biotherapeutic tool is gaining significant importance in the field of medical research [7-8]. The emergence of RNA biotherapeutics including small interfering RNA (siRNA) therapeutics, RNA aptamers and antisense reagents which are all chemically synthesized, has led to the requirement of high throughput chromatographic procedures to purify and characterise RNA. Ion pair reverse-phase chromatography (IP RP HPLC) has been widely applied for the study of nucleic acids, in particular DNA [9-10]. More recently, a number of studies have utilised IP RP HPLC for the purification and analysis of RNA, demonstrating its versatility in a variety of different applications; from the routine purification of synthetic oligoribonucleotides, through to the analysis of complex RNA:RNA interactions. This research demonstrates that IP RP HPLC is a versatile platform for the analysis of RNA. Careful selection of the chromatography conditions including the ion pair reagent, temperature and additives to the mobile phase, facilitates the operation of the IP RP HPLC under different modes, enabling the study of a wide of range RNAs and biological systems.

Ion pair reverse-phase chromatography

Ion pair reverse-phase chromatography is the predominant mode of chromatography that has been widely employed for nucleic acid separations [9-10]. The development of 2 µm, C_{18} surface, non-porous polymeric columns, based on the original work by Bonn et al. [11-14] and later commercialised as the DNASep® column (Transgenomic, USA) enabled the high resolution separations of both double-stranded (ds) and single-stranded (ss) nucleic acids in less than 10 minutes and in many cases resolving fragments differing in only a single base pair. In addition, a number of monolithic polymeric materials have also been utilized for high resolution nucleic acid separations [15, 16]. The ion pairing reagent is an amine cation salt that forms a hydrophobic ion pair with the phosphate anion group of the nucleic acid. Triethylammonium acetate (TEAA) is the most common amine cation salt used for nucleic acid separations. TEAA pairs with nucleic acid fragments to form a hydrophobic ion-pair and adsorb to the hydrophobic surface of the column. Acetonitrile is gradually added to the mobile phase to decrease the polarity of the mobile phase until TEAA / nucleic acid ion pair fragments desorb from the column. An increasing concentration of acetonitrile results in the elution of the smaller fragments first and subsequently fragments increasing in size elute as the acetonitrile concentration is increased.

The mechanism of ion pair reverse-phase chromatography has been discussed and debated in detail since it was first introduced over 30 years ago. A number of alternative names are found in the literature depending on the proposed mechanism and application [17-24 reviewed in 25]. The ion pair model proposed that solute ions form neutral ion pairs with the ion pair reagent in the mobile phase [17-19]. These neutral ion pairs are retained on the hydrophobic stationary phase with enhanced retention. However, an alternative dynamic ion-exchange model proposed that the ion pair reagent first adsorbs at the surface of the stationary phase, providing exchange sites for analyte ions [20-23]. It was also proposed that both ion pairing and dynamic ion exchange occur and that the extent to which one is more significant than the other depends on the experimental conditions [23-24]. Bidlingmeyer and co-workers described the role of the electrical double layer that is formed when the ion pair reagent dynamically adsorbs onto the reverse phase media [23-24]. The applications of ion pair reverse-phase chromatography are diverse, a wide range of compounds have been analysed using this approach [reviewed in 25]. The application of IP RP HPLC for the analysis of RNA will be discussed.

Analysis of single stranded oligoribonucleotides

Depending on the type of mobile phase used and the particular RNA molecules being

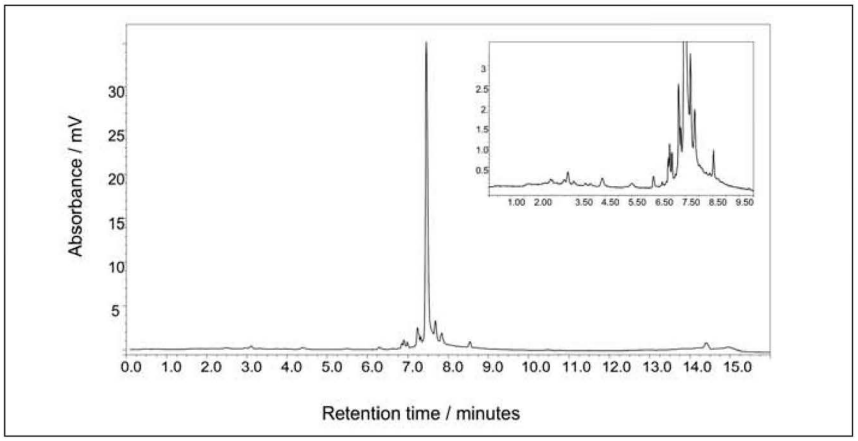

Fig. 1. IP RP HPLC analysis of a synthetic oligoribonucleotide. A 23-mer RNA was separated on a non-porous, alkylated PS(DVB) column using the following gradient. Buffer A 0.1 M TEAA, pH 7.0; Buffer B 0.1 M TEAA, pH 7.0, 25% acetonitrile. The gradient was extended from 20%-22% B in 2 minutes, 22-52% B over 15 minutes, 52-65% B over 2.5 minutes at a flow rate of 1.0 ml/min at 75 °C. Inset shows an enhanced view of the short-mer and long-mer impurities present.

separated, single-stranded (ss) nucleic acid separations are based on differences in size, hydrophobicity (sequence) and conformation. The separation of short oligoribonucleotides (RNA) is essentially similar to the separation of short oligodeoxyribonucleotides (DNA) using IP RP HPLC. The hydrophobic properties of the stationary phase depends on the number of methyl groups in the alkyl chain of the ion pair reagent. Short alkyl chains such as those in TEAA, allow the stationary phase to partially retain its hydrophobic or reverse phase properties as TEAA only partially covers the stationary phase. Therefore, separation is dependent on both size and base composition. The four different nucleotide bases in RNA are adenine (A), uracil (U), cytosine (C) and guanine (G) which differ in their relative hydrophobicity. As C is less hydrophobic compared to A, C rich sequences elute prior to A rich RNA sequences when an acetonitrile gradient is applied. The effect is often greater if the nucleotide is positioned at the end(s) of the fragment [11, 13]. This sequence dependent effect can be reduced or eliminated for single-stranded RNA separations if a more hydrophobic ion pair reagent, such as tetrabutylammonium bromide (TBAB) or n-hexylammonium acetate ion is used instead of the more commonly used TEAA. The longer alkyl chains provide complete coverage of the stationary phase and a dynamic anion-exchange is the predominant mechanism, resulting in size based separation with no sequence effects.

With the emergence of RNA biotherapeutics, including siRNA therapeutics, RNA aptamers and antisense reagents which are all chemically synthesized, the requirement of high throughput, robust approaches to isolate the RNA from impurities such as failure sequences (typically n-1 n-2 also termed short-mers) and long-mers (typically containing deprotecting groups) is paramount. A wide range of studies have been developed centred on IP RP HPLC in conjunction with range of different reverse phase media for the separation and purification of synthetic oligoribonucleotides, including siRNAs [26-30] and chimeric ribozymes [31]. Recently IP RP UPLC analysis of synthetic oligoribonucleotides (siRNAs) was performed [28-30]. A typical IP RP HPLC chromatogram observed for the analysis of synthetic oligoribonucleotide is shown in Figure 1. The chromatogram shows the presence of failure sequences/degradation products and long-mers which are typical removed following purification.

The high resolution separation of short oligoribonucleotides was further developed for the analysis of RNA footprinting studies to analyse the solvent accessibility of the hairpin ribozyme [32]. High resolution separation of fluorescently labeled RNAs generated from the base catalysed hydrolysis of a fluorescently labeled synthetic RNA corresponding to the substrate strand of the hairpin ribozyme is shown in Figure 2. The use of fluorescent labeling enabled both separation and direct quantitation of the cleavage products generated from hydroxyl radical footprinting reactions [32]. Using the ion pair reagent tetrabutylammonium bromide single nucleotide resolution of the base catalysed FAM labeled RNA products was achieved by virtue of sequence dependent separations with little or no influence from the hydrophobic fluorescent group. A number of alternative fluorescent based assays utilising these sequence based separations of fluorescently labeled RNA have been performed including the analysis of RNA cleavage reactions catalysed by Escherichia coli RNase HII [33], Escherichia coli Ribonuclease E [34], a DNAzyme generated through in vitro selection [35], the analysis of oligoribonucleotide-spermine conjugates [36] and the kinetic analysis of the Varkus Satellite (VS) ribozyme [37]. These IP RP HPLC assays enabled simultaneous separation and quantitation of the reaction products to obtain catalytic parameters. These studies have demonstrated that IP RP HPLC fluorescent based assays offer significant advantages

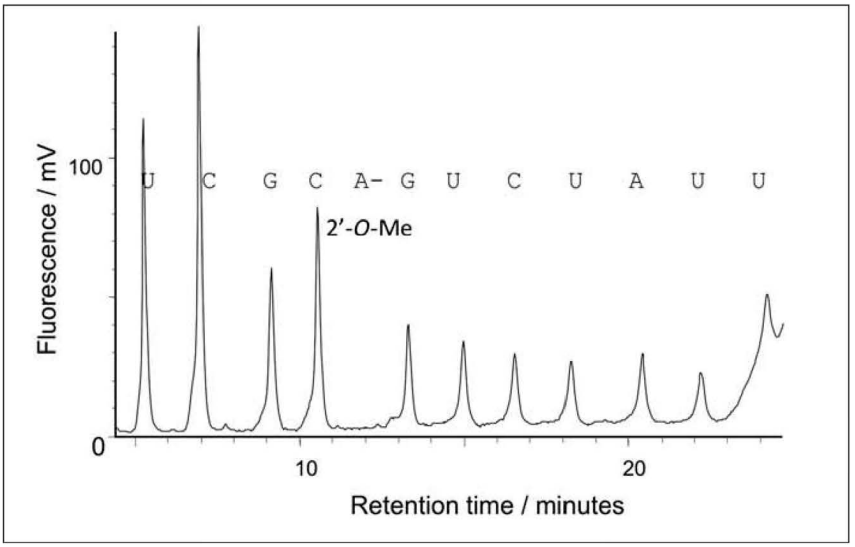

Fig. 2. Base catalysed hydrolysis of fluorescently labeled RNA. IP RP HPLC profile generated from the base catalysed hydrolysis of the fluorescently labelled hairpin ribozyme substrate strand analysed on a non-porous, alkylated PS(DVB) column using the following conditions. Buffer A 0.0025 M TBAB , 0.1% acetonitrile, 1 mM (Na_4) EDTA, Buffer B 0.0025 M, TBAB, 70% acetonitrile,1 mM (Na4) EDTA. Starting at 25% buffer B the gradient was extended to 42% buffer B over 10, followed by an extension to 50% buffer B over 15 minutes at a flow rate of 0.9 ml/min. No cleavage is seen at the (a-1) position which contains a 2'-O-methyl group that is resistant to base catalysed hydrolysis. Reproduced with permission from [32].

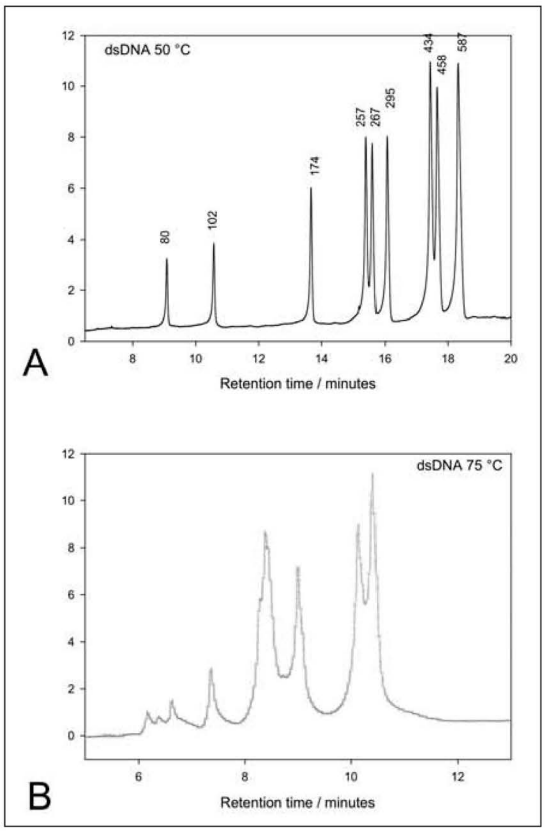

Fig. 3. IP RP HPLC analysis of dsDNA and ssDNA. (A) pUC18 HaeIII digest at 50 °C (B) pUC18 HaeIII digest at 75 °C. The size of the nucleic acid fragments in nucleotides/base pairs are highlighted. The DNA was analysed using the following gradient. Buffer A 0.1 M TEAA, pH 7.0; Buffer B 0.1 M TEAA, pH 7.0, 25% acetonitrile. 10%-20% B in 2.5 minutes, 20-40% in 2.5 minutes, 40-70% B in 13 minutes at a flow rate of 1.0 ml/min at 75 °C. Reproduced with permission from [46]

compared to traditional radiolabeling of the RNA in conjunction with denaturing gel electrophoresis and densitometry analysis for the separation and quantitiation of RNA cleavage products.

Interfacing IP RP RNA chromatography with mass spectrometry analysis

Mass spectrometry has emerged as an increasingly powerful tool for the identification and characterisation of nucleic acids. With the increasing use of synthetic RNAs as biotherapeutic drugs, rigorous analysis and characterisation is required to meet regulatory demands. The interfacing of IP RP µHPLC to mass spectrometry analysis offers a powerful tool to aid in RNA analysis and characterisation. Traditional ion pair reagents such as TEAA and TBAB are not compatible with mass spectrometry analysis due to significant ion suppression observed at typical ion pair concentrations that enable high resolution RNA separations. There is often a trade off with lower chromatographic performance with enhanced MS sensitivity as the concentration of ion pair reagent is reduced in the mobile phase. A significant development of a suitable mobile phase that was MS compatible was introduced by Apffel et al. 1997 with the addition of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as an additive to the HPLC mobile phase [38] and has since been further developed and utilised in a variety of applications [39-40]. In addition, the replacement of the TEAA ion pair reagent with more volatile ion pair reagents such as triethylammonium bicarbonate (TEAB) [16] butyldimethylammonium bicarbonate (BDMAB)/butyldimethylammonium acetate [27, 41] and cyclohexyldimethylammonium acetate (CycHDMAA) [42] have all successfully been used for nucleic acid separations interfaced with mass spectrometry analysis. Monolithic, poly(styrene–divinylbenzene)-based capillary columns have been utilised for nucleic acid separations interfaced to mass spectrometry using the described MS compatible mobile phases in a number of different applications [reviewed in 15]. Using this approach mass spectrometry analysis of synthetic (siRNA) and biological RNAs (transfer RNA and 5S rRNA) enabled the generation of sequence information of the oligoribonucleotide from low picomole quantities [27]. More recently, IP RP UPLC RNA separations interfaced to MS analysis has also been performed in conjunction with the TEA/HFIP mobile phase and UPLC OST C_{18} columns for the analysis and sequencing of synthetic oligoribonucleotides [30].

Analysis of large RNA fragments

The rapid high resolution separation of dsDNA fragments on non-porous alkylated resins under ion pair reverse-phase conditions results in predominantly size based separation of the duplex DNA fragments in conjunction with TEAA ion pair reagent [13, 43]. Further work demonstrated the ability of IP RP HPLC to accurately size AT rich dsDNA fragments that migrate anomalously during gel electrophoresis and ion exchange chromatography [43]. This work demonstrates the advantages for the accurate sizing of dsDNA fragments on non-porous alkylated resins under ion pair reverse phase conditions over alternative electrophoretic and chromatographic modes [43]. The ability to separate larger RNA molecules using IP RP HPLC with high resolution, similar to that achieved for larger dsDNA molecules was demonstrated in the development of an assay for group I intron ribozyme activity [44] . Further studies have utilised the rapid high resolution separation of RNA to analyse a wide range of RNA transcripts and biological RNAs including, ribosomal RNA and mRNA [45-47]. IP RP HPLC was also used for the rapid separation and fractionation of tRNA, small rRNA and large rRNA species in a single chromatographic analysis [47]. This study was also extended in the analysis of tRNA, small rRNA and large rRNA transcripts using capillary chromatography in conjunction with monolithic, poly(styrene–divinylbenzene)-based capillary columns [47].

The ability of RNA chromatography to resolve closely related 16S rRNA sequences was also demonstrated in the analysis of E.coli and P.putida 16S rRNA (1534 vs 1518 nt). Following extraction of the 16S rRNA, RNA chromatography was used to readily distinguish the two different 16S rRNAs species by virtue of differences in the retention time [46]. Enhanced sensitivity in the detection of RNA using IP RP HPLC has been achieved by using post column intercalation of fluorescent dyes in conjunction with fluorescent detection of the RNA [47]. The incorporation of an unsymmetrical cyanine dye (SYBR gold) that binds to RNA and enables fluorescent based detection of the RNA enabled a 1000-fold increase in sensitivity compared to traditional UV absorption and permitted the detection of pg quantities of ssRNA using this approach [48].

The high resolution separation of these larger ssRNA fragments was surprising, considering the low resolution separation of larger ssDNA molecules using IP RP HPLC. We have further investigated the mechanism by which high resolution separation of large RNA fragments using IP RP HPLC is achieved. We analysed duplex RNA and DNA fragments at both 50 °C and 75 °C [46]. The analysis of a range of dsDNA fragments of different sizes generated from a pUC18 HaeIII DNA digest at elevated temperatures resulted in a significant loss of resolution of the ssDNA fragments compared to the high resolution separation of the duplex DNA at 50 °C (see Figure 3A/B). In a similar manner to dsDNA, the high resolution separation of large dsRNA fragments (80-1000 bp) was also observed (see Figure 4A). However, even at 75 °C the high resolution of

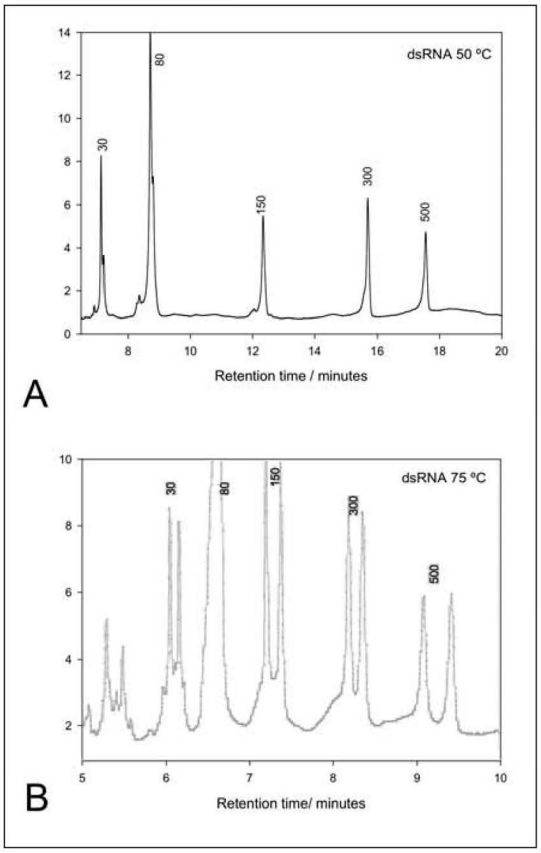

Fig. 4. IP RP HPLC analysis of dsRNA and ssRNA at 50 °C. (A) dsRNA marker at 50 °C (B) dsRNA marker at 75 °C. The size of the nucleic acid fragments in base pairs/nucleotides are highlighted. The samples were analysed using the following gradient . Buffer A 0.1 M TEAA, pH 7.0; Buffer B 0.1 M TEAA, pH 7.0, 25% acetonitrile. 10%-20% B in 2.5 minutes, 20-40% B in 2.5 minutes, 40-70% B in 13 minutes at a flow rate of 1.0 ml/min at 50 °C. Reproduced with permission from [46]

the ssRNA was obtained, in contrast to that observed for ssDNA (see Figure 4B). It was proposed that the ssRNA maintains duplex/secondary structures even at elevated temperatures (75 °C) that permits the high resolution separation. Each of the duplex dsRNA strands was resolved into two ssRNA species at 75 °C, demonstrating that the separation of the ssRNA is no longer size dependent but is dependent on both size and sequence reflecting the degree of duplex structure and overall hydrophobicity of the RNA molecule [46].

The proposed presence of secondary structure elements within the RNA was utilised in the analysis and purification of oligoribonucleotide stem loop structures at 30 °C in the presence of TEAA [49]. In addition, the proposed presence of secondary structure elements was further investigated and utilised in the development of a chromatographic assay to study RNA:RNA interactions. The RNA component of human telomerase (hTR, 451 nt) was in vitro transcribed and analysed using IP RP HPLC in a temperature dependent manner in conjunction with the incorporation of Mg^{2+} ions into the mobile phase [46]. The presence of Mg^{2+} ions stabilised the dimeric (or multimeric) hTR RNA species, resulting in this species eluting later than the monomer (see Figure 5). The early eluting RNA species (a) when analysed using polyacrylamide gel electrophoresis (PAGE) migrates as the monomer and the later peak (b) migrates as the multimeric species. These results demonstrate that in the presence of Mg^{2+} ions the dimer (or multimeric) hTR RNA species, is stabilised and elutes later than the monomer. These results were consistent with the observations using native PAGE and agarose gel electrophoresis that hTR can dimerise in vitro [50, 51]. By incorporating magnesium ions and low temperatures, the chromatography allows the stabilisation of RNA:RNA interactions in hTR and such complexes can be resolved on the basis of differential hydrophobicity in a temperature dependent manner. The ability of IP RP HPLC to examine the conformation of RNA was also demonstrated in the analysis of the 124nt long BRCA1 Ex1a transcript. Sobczak and Krzyzosiak showed that this molecule was capable of adopting alternative stable RNA conformations using both non-denaturing PAGE and capillary electrophoresis-laser induced fluorescence (CE-LIF) (following fluorescent end labeling of the RNA transcript) [52]. Following transcription and analysis of the RNA, IP RP HPLC was used to confirm the structural heterogeneity of the transcript using non-denaturing PAGE (see Figure 6). Figure 6A shows the appearance of two bands indicative of multiple conformations. Analysis of the Ex1a transcript was also performed using IP RP HPLC (see Figure 6B) and reveals the presence of multiple peaks in the chromatogram, consistent with the formation of several stable conformers.

Summary

Ion pair reverse-phase chromatography in conjunction with non-porous polymeric columns and monolithic polymeric capillary columns has emerged as a versatile platform for the analysis of RNA. High resolution separations of both double-stranded and single-stranded RNA can be achieved in less than 10 minutes. The use of RNA chromatography not only provides an efficient means of isolating and analysing RNA species which is of particular interest in the purification and characterisation of RNA biotherapeutics, it can also provide valuable structural information. Combined with structural studies such approaches will provide further insight in the analysis of RNA. In contrast to the analysis of large ssDNA fragments the high resolution separation of large ssRNA is observed at elevated temperatures. Secondary structure elements survive in the RNA fragments at elevated

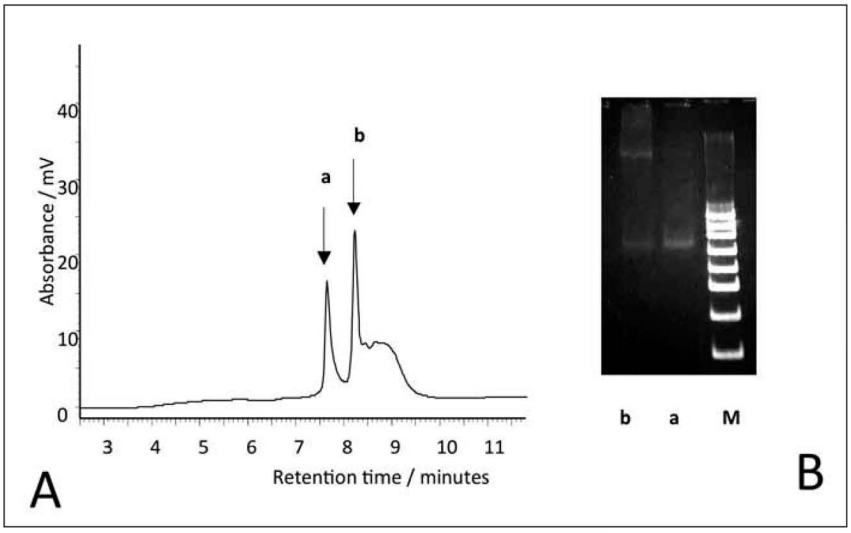

Fig. 5. Electrophoretic and chromatographic analysis of hTR. (A) IP RP HPLC chromatogram of hTR in the presence of 1 mM Mg2+ at 40 °C. The RNA was analysed using the following gradient. Buffer A 0.1 M TEAA, pH 7.0 1 mM Mg2+ Buffer B 0.1 M TEAA, pH 7.0 1 mM Mg2+, 25% acetonitrile. 30% -60% B in 10 minutes at flow rate of 1.0 ml/min. The hTR species (a) and (b) were fractionated using and analysed using PAGE. (B) Electrophoretogram showing the analysis of the fractionated hTR samples. Lane M contains a 100 bp DNA ladder. Reproduced with permission from [46].

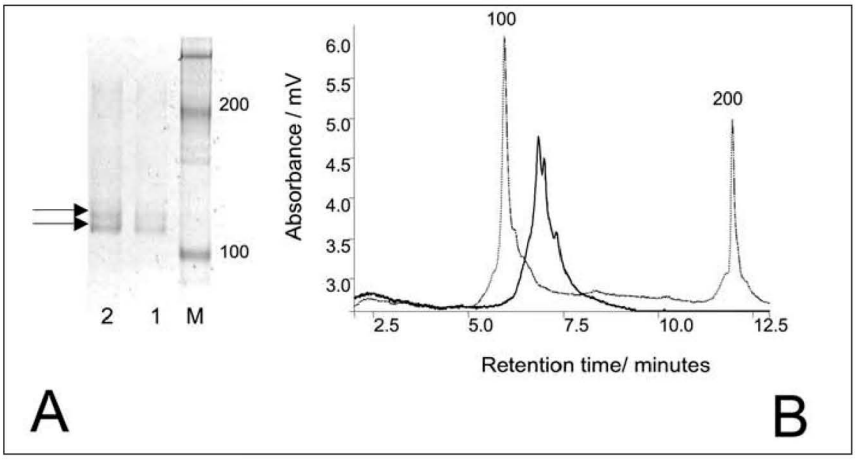

Figure 6. Analysis of BRCA1 RNA transcripts. (A) 6% non-denaturing PAGE of the Ex1a transcript (124nt) following folding in the presence of 10mM Mg^{2+}. Lane 2 contains 2X concentration of RNA used in lane 1. Lane M contains an RNA marker (100-1000nt) (B) IP RP HPLC analysis of the Ex1a transcript (solid line) and RNA Marker (dashed line) using the following gradient. Buffer A 0.1 M TEAA, pH 7.0; buffer B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile. Starting at 25% buffer B the gradient was extended to 30% buffer B in 1 minute, followed by an extension to 45% buffer B over 15 minutes at a flow rate of 0.9 ml/min.

temperatures, leading to enhanced resolution separation in a pseudo-size dependent fashion. The relative hydrophobicity of the RNA fragments is a reflection of both the secondary structure present and the nucleic acid sequence. Alternative conformations of RNA molecules present different hydrophobic surfaces to the stationary phase and therefore allow the chromatographic resolution of such molecules. Manipulation of the chromatography conditions including the ion pair reagent, temperature and additives to the mobile phase, facilitates the operation of the IP RP HPLC under different modes, enabling the study of a wide range of RNAs and biological systems.

Acknowledgments

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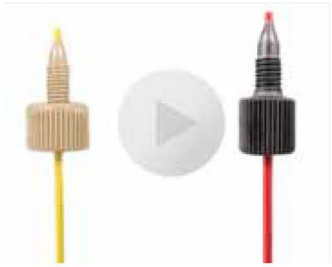
How to Maximise HPLC Results with High-Performance Media



Phenomenex (USA) has produced an online video tutorial on optimising conventional HPLC systems for use with advanced, high-performance media. New core-shell technology columns, such as Kinetex® from Phenomenex, can produce ultra-high performance results on standard LC systems. Extra dead volume in HPLC instruments prevents maximum performance of the new, high-performance columns. With easy HPLC system modifications, Kinetex users can realise performance improvements of up to 130%. The Phenomenex video tutorial demonstrates simple ways to minimise this dead volume, such as using smaller-diameter connective tubing and switching to a low-volume needle seat and detector flow cell.

"We developed and produced this tutorial to help chromatographers realise maximum potential from the new column technology on existing systems," commented Jeremy Bierman, Brand Manager for Phenomenex. "Recent studies have shown that Kinetex core-shell columns are redefining what is possible in HPLC and that improvements to even the best instruments must be made before the full potential of these new high-performance columns is realised."

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