Separation of RNA Phosphorothioate Oligonucleotides by HPLC

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CHAPTER FOURTEEN

Separation of RNA Phosphorothioate Oligonucleotides by HPLC

John K. Frederiksen[*,†,1] *and* Joseph A. Piccirilli[†,‡]

Contents

Abstract

Phosphorothioate oligonucleotides are indispensable tools for probing nucleic acid structure and function and for the design of antisense therapeutics. Many applications involving phosphorothioates require site- and stereospecific substitution of individual *pro-*R_P or *pro-*S_P nonbridging oxygens. However, the traditional approach to phosphorothioate synthesis produces a mixture of R_P and S_P diastereomers that must be separated prior to use. High-performance

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liquid chromatography (HPLC) has proven to be a versatile method for effecting this separation, with both reversed phase (RP) and strong anion exchange (SAX) protocols yielding favorable results. In this chapter, we present several examples of successful separations of RNA phosphorothioate diastereomers by HPLC. We also report the use of complementary DNA oligonucleotides for the separation of poorly resolved phosphorothioate RNAs.

1. Introduction: Phosphorothioate Oligonucleotides and the Need for Separation

The unique properties of phosphorothioate oligonucleotides make them suitable for a wide range of biochemical and medicinal applications. In a phosphorothioate, sulfur replaces one of the two nonbridging oxygens of a phosphodiester linkage. This substitution confers chirality at the phosphorous center (Fig. 14.1) and shifts the electronic charge distribution of the linkage so that the formal negative charge localizes on sulfur (Frey and Sammons, 1985). The bulkier sulfur atom and the longer phosphorous–sulfur bond also alter the steric environment from that of the original phosphodiester linkage. As a result of these changes, biological macromolecules often interact preferentially with one of the two diastereomers,

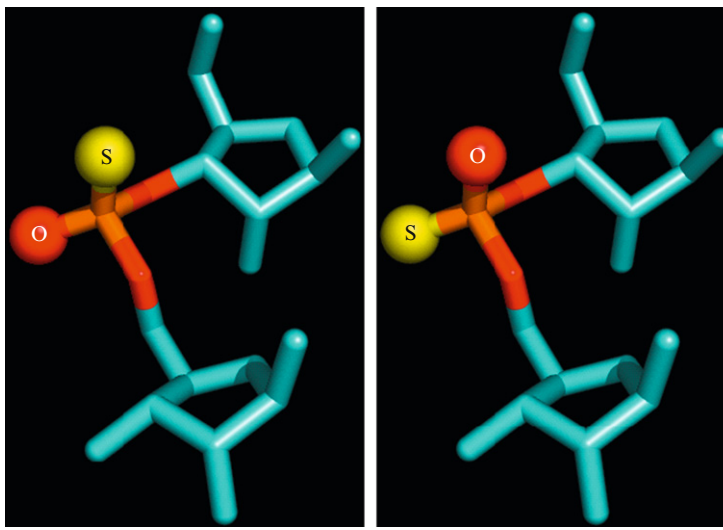


Figure 14.1 RNA phosphorothioate diastereomers. Sulfur sizes and bond lengths not drawn to scale.

making phosphorothioates useful as mechanistic and structural probes. Several papers have reported the use of phosphorothioates to determine the stereochemical course of protein- and RNA-catalyzed reactions (Burgers *et al.*, 1979; Eckstein *et al.*, 1972, 1981; McSwiggen and Cech, 1989; Moore and Sharp, 1993; Padgett *et al.*, 1994; Potter *et al.*, 1983a). Moreover, as sulfur preferentially coordinates softer metal ions than does oxygen, phosphorothioates have figured prominently in the investigations of metal ion catalysis in ribozymes via the metal ion rescue approach (Chen *et al.*, 1997; Christian *et al.*, 2000, 2006; Crary *et al.*, 2002; Forconi *et al.*, 2008; Gordon and Piccirilli, 2001; Hougland *et al.*, 2005; Osborne *et al.*, 2005; Shan *et al.*, 2001; Wang *et al.*, 1999; Warnecke *et al.*, 1996; Yoshida *et al.*, 1999; see also Chapter 15 in this volume). In the context of DNA, phosphorothioates confer resistance to nucleases and are incorporated into therapeutic antisense oligonucleotides to increase plasma half-life and improve pharmacokinetics (Akhtar *et al.*, 1991; Hoke *et al.*, 1991; Stein and Cheng, 1993). In addition to these biochemical applications, phosphorothioates also appear to play a physiologic role for some organisms. Recent work has demonstrated that certain bacteria incorporate phosphorothioates within their genomes, which may help to maintain the structural integrity of the bacterial chromosome (Wang *et al.*, 2007).

Many experiments involving phosphorothioates, especially those investigating catalytic reaction mechanisms, require site- and stereospecific phosphorothioate substitutions. An example encountered frequently in the field of ribozyme catalysis is the sulfur substitution of a *pro*-R_P or *pro*-S_P nonbridging oxygen suspected of coordinating a catalytic metal ion. The nonbridging oxygens are not equivalent stereochemically and must be substituted individually with sulfur to assess their potential catalytic roles. To this end, diastereomerically pure R_P and S_P phosphorothioate substrates or ribozymes must be constructed. Depending on the experiment, this can entail sequential splint-mediated enzymatic ligations of short, diastereospecific phosphorothioate oligonucleotides to *in vitro*-transcribed flanking RNAs (Moore and Sharp, 1993; Stark *et al.*, 2006; see also Chapter 2 of volume 469; Fig. 14.2).

One of the challenges associated with obtaining diastereomerically pure phosphorothioates is the method by which phosphorothioates are introduced during solid-phase oligonucleotide synthesis. In each synthetic cycle, the free 5′-OH of the immobilized oligonucleotide attacks the phosphorous atom of an incoming phosphoramidite (Fig. 14.3). To form the natural phosphodiester, the resulting trivalent phosphite is oxidized to the pentavalent phosphate by treatment with a mixture of iodine and water. In phosphorothioate synthesis, the phosphite is treated instead with one of several different sulfurization agents (Connolly *et al.*, 1984; Iyer *et al.*, 2002; Song *et al.*, 2003; Stec *et al.*, 1993; Vu and Hirschbein, 1991; Xu *et al.*, 1996). This oxidizes P(III) to P(V) as in standard synthesis, but with sulfur replacing one of the nonbridging oxygens, producing a mixture of R_P and S_P

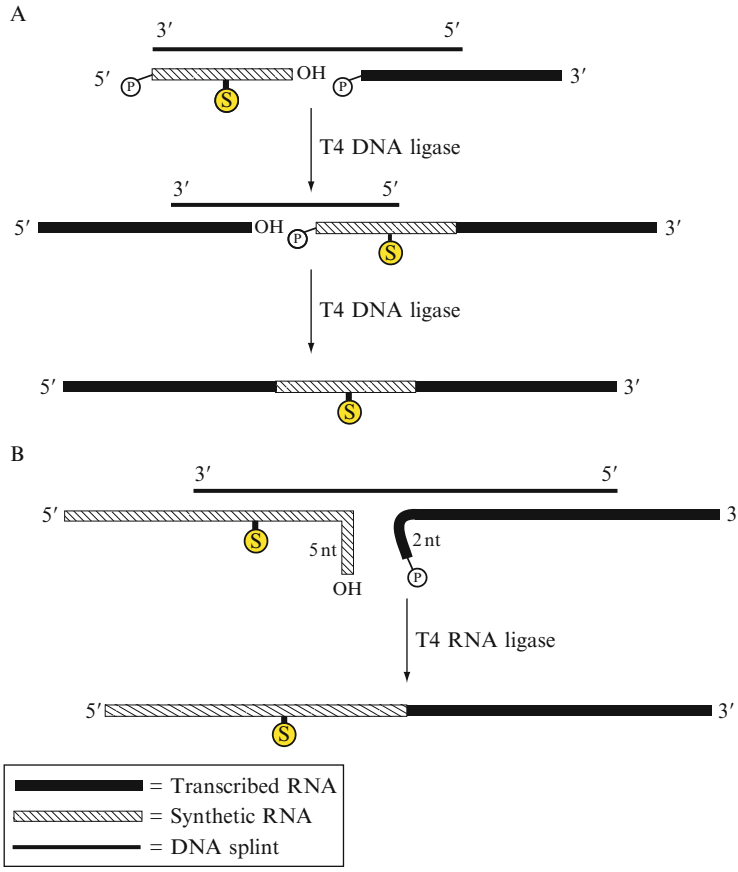
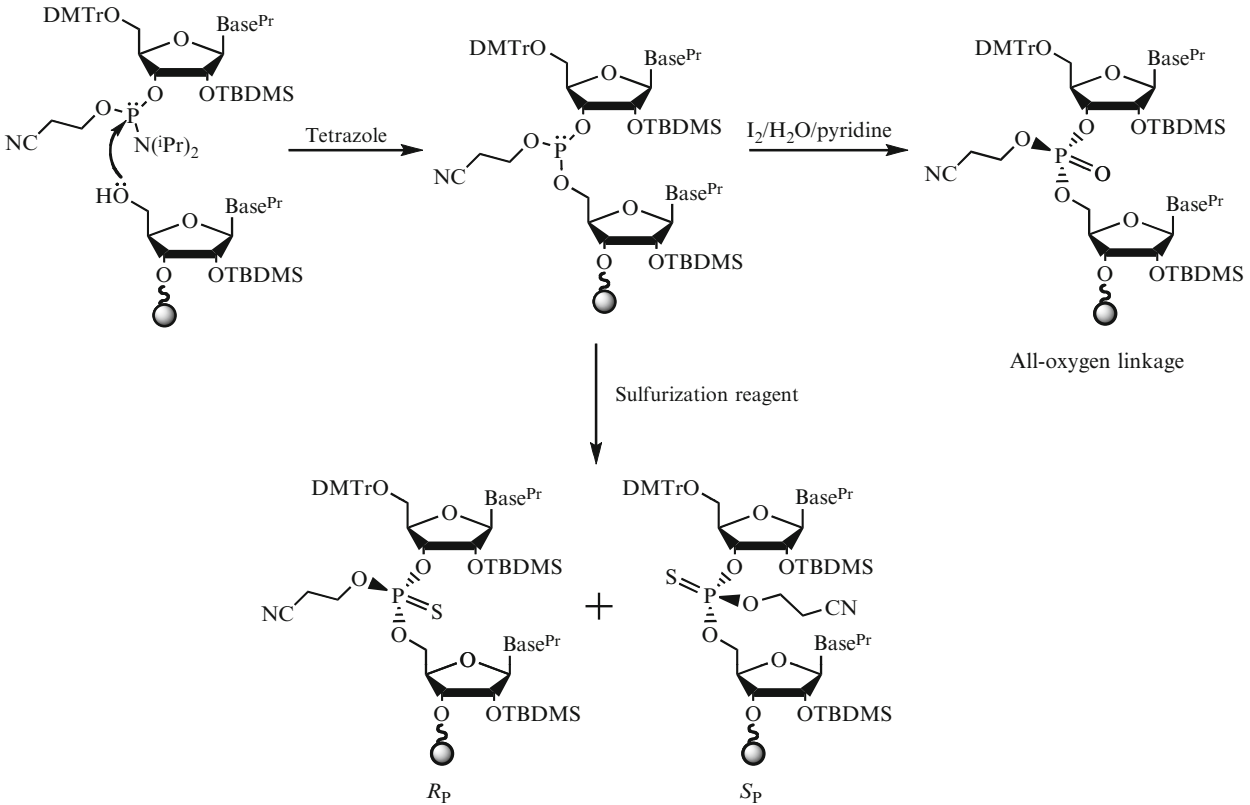


Figure 14.2 Semisynthetic construction of RNAs containing site- and diastereospecific phosphorothioate substitutions. (A) T4 DNA ligase-mediated ligation (Moore and Sharp, 1993). The RNAs to be joined together are held in apposition by a fully complementary bridging DNA splint. T4 DNA ligase recognizes the duplex as nicked, and catalyzes the attack of the free 3′-OH on the 5′-phosphate group. This reaction proceeds with relatively low turnover due to the slow rate of dissociation of the enzyme from the duplex. Consequently, preparation of large quantities of modified RNA often requires near-stoichiometric quantities of ligase. (B) T4 RNA ligase-mediated ligation (Stark *et al.*, 2006). For single-stranded DNA or RNA, this enzyme catalyzes the attack of any free 3′-OH on any 5′-phosphate group with multiple turnover. In ligation reactions, the RNAs to be joined together are held in place with a bridging DNA splint that places the ligation junction within a seven-nucleotide bulge. This bulge includes the last five nucleotides and free 3′-OH of the 5′-RNA, and the 5′-phosphate group and first two nucleotides of the 3′-RNA. T4 RNA ligase recognizes the free ends of the bulge as single stranded and ligates them together. To minimize cross reactions between the splint and 5′-phosphorylated RNA, these ligations may be performed with DNA splints containing a 3′-dideoxynucleotide.


DMTrO
Base^Pr
OTBDMS
NC
N(iPr)2
HO
Tetrazole
I2/H2O/pyridine
All-oxygen linkage
Sulfurization reagent
S
CN
RP
SP
Mixture of diastereomeric phosphorothioates

diastereomers that must be separated. Methods of introducing diastereospecific phosphorothioates do exist and have been adapted for use in automated synthesizers (Guga and Stec, 2003; Oka *et al.*, 2008, 2009; Fig. 14.4). However, these methods require the synthesis of new *P*-chiral monomer units and have not yet replaced the traditional approach outlined above. In time, they should prove instrumental for routine synthesis not only of single stereospecific phosphorothioates, but also of multiple such linkages within a single oligonucleotide. This is not easily achievable using standard synthetic protocols, since the introduction of each new phosphorothioate results in a more complex mixture of diastereomers whose separation becomes intractable.

2. HPLC Separation of Phosphorothioate Diastereomers

Once synthesis and deprotection of a phosphorothioate oligonucleotide is completed, HPLC is the method of choice for separation of the R_P and S_P diastereomers. Several examples exist in the literature for reversed phase (RP) HPLC separation of DNA phosphorothioates (Murakami *et al.*, 1994; Stec and Zon, 1984; Stec *et al.*, 1984, 1985). For RNA phosphorothioates, the work of Slim and Gait on synthetic hammerhead ribozyme substrates is often cited (Slim and Gait, 1991). This involved the synthesis and purification of a 13-nt RNA substrate containing a stereospecific phosphorothioate at the hammerhead cleavage site. The substrate was purified using both strong anion exchange (SAX) and RP HPLC protocols. With SAX using a potassium phosphate buffer system as an initial purification step, the diastereomers eluted as a single peak. In contrast, the R_P and S_P diastereomers eluted as two peaks separated by about 1 minute using RP HPLC with 0.1 *M* ammonium acetate and an increasing percentage of acetonitrile. Subsequent variations on this protocol, as well as Tris- and ammonium acetate-based SAX buffer systems, have proved successful for separating phosphorothioates of many different lengths and sequences (Table 14.1).

The need for multiple protocols and buffer systems underscores that while some general principles of HPLC may be applied to phosphorothioate

Figure 14.3 Solid-phase synthesis of phosphorothioate oligonucleotides. Following tetrazole-catalyzed addition of the incoming nucleoside phosphoramidite, treatment with a mixture of iodine and water in pyridine oxidizes the phosphite intermediate to form a phosphotriester linkage. Subsequent base-catalyzed removal of the β-cyanoethyl protecting group yields the natural phosphodiester (not shown). In phosphorothioate synthesis, a sulfurization reagent effects oxidation, producing a mixture of phosphorothioate diastereomers. Abbreviations: DMTr, dimethoxytrityl; $Base^{Pr}$, chemically protected nucleobase; TBDMS, *tert*-butyldimethylsilyl; $N(^iPr)_2$, *N,N*-diisopropylamino.

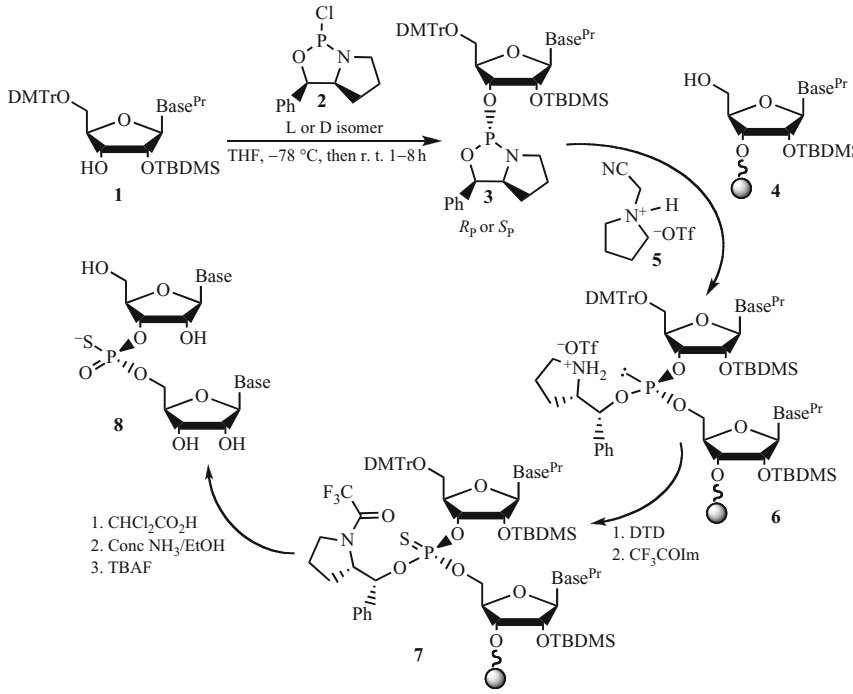


Figure 14.4 Synthesis of diastereospecific RNA phosphorothioates. *P*-chiral monomer units in the form of 3′-*O*-oxazaphospholidines (3) were synthesized by reacting 5′-DMTr- and 2′-TBDMS-protected nucleosides (1) with a stereospecific 2-chloro-1,3,2-oxazaphospholidine derivative **2**. The monomers were coupled to nucleosides attached to a solid support (4) in the presence of the activator *N*-(cyanomethyl)pyrrolidinium triflate (CMPT) (5). Treatment of the resulting intermediate **6** with *N,N'*-dimethylthiuram disulfide (DTD) yielded the diastereospecific phosphorothioate. Acetylation of unreacted 5′-hydroxyl groups was accomplished with *N*-trifluoroacetylimidazole (CF_3COIm), which also acetylated the secondary amino group derived from CMPT to produce **7**. Subsequent detritylation, cleavage from the solid support, and removal of TBDMS groups with tetra-*n*-butylammonium fluoride (TBAF) gave the free phosphorothioate oligonucleotide **8**. Adapted from Oka *et al.*, 2008, 2009.

separations, establishing optimal conditions remains an empirical process. A successful separation depends on a multitude of variables—oligonucleotide length and sequence, phosphorothioate position, column type, buffer composition, slope of the elution gradient, and temperature, to name just a few. Consequently, whether and to what extent separation will occur cannot be determined with any degree of certainty, aside from the prediction that the phosphorothioates will have a longer retention time than the corresponding all-oxygen oligonucleotide. In our experience, separation of a seemingly inseparable phosphorothioate requires patience and a low threshold for switching to alternative HPLC conditions.

Table 14.1 Published HPLC conditions for separation of phosphorothioate oligoribonucleotides

Strong anion exchange		
Buffers	**Length (nt)**	**Reference**
A: 0.025 *M* tris-HCl pH 8.9 B: 0 to 900 m*M* NaCl	20	Hougland *et al.* (2005)
A: 0.010 *M* tris-HCl pH 9.3, 10% EtOH B: 20 to 1000 m*M* NaCl	17 19, 22	Wang *et al.* (1999) Gordon and Piccirilli (2001)
A: 0.1 *M* NH_4OAc, 2% CH_3CN, pH 8 B: 0 to 900 m*M* KCl	15, 16	Dertinger *et al.* (2000)
A: 0.025 *M* tris-HCl pH 7.4 B: 0 to 900 m*M* NaCl	8, 15	Forconi *et al.* (2008)

Reversed phase		
Buffers	**Length (nt)**	**Reference**
A: 0.1 *M* NH_4OAc B: 60% 0.1 *M* NH_4OAc/ 40% CH_3CN	7	Yoshida *et al.* (1999)
A: 0.1 *M* NH_4OAc	11	Christian *et al.* (2000), Christian *et al.* (2006)
B: 20% A/80% CH_3CN	13 17	Slim and Gait (1991) Peracchi *et al.* (1997), Wang *et al.* (1999)
A: 0.1 *M* NH_4OAc pH 7 B: CH_3CN	15, 16 19, 22	Dertinger *et al.* (2000) Gordon and Piccirilli (2001)
A: 0.1 *M* TEAAc pH 7 B: 20% 0.1 *M* TEAAc pH 7/80% CH_3CN	13	Warnecke *et al.* (1996)
A: 20 m*M* NH_4OAc pH 7, 2% CH_3CN B: 20 m*M* NH_4OAc pH 7, 20% CH_3CN	10	Crary *et al.* (2002)

2.1. Biochemical characterization

Basic biochemical characterization of a newly synthesized phosphorothioate prior to separation can save much time and vexation at the HPLC. On more than one occasion, we have tried to separate a particular phosphorothioate without success, only to discover subsequently that the oligonucleotide did not contain sulfur. Simple assays like alkaline hydrolysis, digestion with one or more RNases to assess sequence, and treatment with iodine to determine the position of the phosphorothioate linkage, can help avoid unnecessary optimization of HPLC conditions.

2.2. Phosphorothioate position

The position of the phosphorothioate within the oligonucleotide can influence the degree of separation achievable by HPLC. Generally, we have found that placing the phosphorothioate toward the center of the oligonucleotide tends to facilitate separation, although this is hardly a rigorous stipulation (see examples below). Some references report better resolution of DNA phosphorothioates when the phosphorothioate is located toward the 5′-end and the 5′-dimethoxytrityl (DMTr) protecting group is left in place (Stec and Zon, 1984; Zon, 1990). In RP HPLC, retaining the hydrophobic 5′-DMTr group increases the retention time of the full-length oligonucleotide, helping to differentiate it from the shorter side products associated with incomplete phosphoramidite coupling. While we have not tested this strategy for RNA phosphorothioates, the examples we present herein suggest that centrally and 3′-end-directed phosphorothioates can be readily separated.

2.3. Elution order

With regard to the order of elution from the column, the R_P diastereomer usually elutes before the S_P diastereomer on both SAX and RP HPLC. This observation has been made for diastereomers of both DNA (Bartlett and Eckstein, 1982; Potter *et al.*, 1983b; Romaniuk and Eckstein, 1982; Stec and Zon, 1984; Stec *et al.*, 1984; Uznanski *et al.*, 1982) and RNA (Burgers and Eckstein, 1978; Crary *et al.*, 2002; Peracchi *et al.*, 1997; Slim and Gait, 1991) phosphorothioates, and is often cited as empirical evidence of the correct assignment of configuration. This convention is not strictly rigorous, however, and in the case of DNA phosphorothioates, the elution order may be reversed if the 5′-DMTr group is retained to facilitate separation (Stec and Zon, 1984; Stec *et al.*, 1984). Ideally, the precise determination of configuration should be made on the basis of enzymatic digestion assays. In particular, R_P phosphorothioates are resistant to cleavage by nuclease P1

(Potter *et al.*, 1983b), while S_P phosphorothioates are resistant to cleavage by snake venom phosphodiesterase (SVP) (Burgers and Eckstein, 1978).

In our laboratory, we have separated many different phosphorothioate RNAs ranging in length from 6 to 25 nucleotides, using both SAX and RP HPLC. The remainder of this chapter describes several examples of these separations, along with their corresponding buffers and gradients. In addition, we present an example of how complementary DNA oligonucleotides can facilitate the separation of poorly resolved phosphorothioates.

3. Materials and Methods

3.1. Oligonucleotide sample preparation

Phosphorothioate oligonucleotides were purchased from Dharmacon, Inc. (Lafayette, CO) and deprotected according to the manufacturer's instructions. The lyophilized pellets were resuspended in 100 μL of water to a concentration of approximately 1–4 m*M*. For test runs, 500–1000 pmol of phosphorothioate oligonucleotide were mixed with water in a final volume of 100 μL and loaded onto the HPLC.

3.2. HPLC hardware

All separations were performed on either a Waters 600 or 2795 HPLC pump, connected to a Waters 996 or 2996 photodiode array, respectively. The pumps were controlled through a single computer running Waters Empower Pro software, which allowed both pumps to operate simultaneously. Oligonucleotide elution from the columns was followed by monitoring the absorbance of the eluent at 260 nm.

3.3. Columns

Separations by SAX employed a semipreparative Dionex DNAPac PA-100 9 × 250 mm anion exchange column running at a flow rate of 2 mL/min. For RP separations, a VyDAC small pore analytical C18 column (201SP54, 4.6 × 250 mm) was used at a flow rate of 1 mL/min. Although not described herein, some large-scale RP separations have been accomplished satisfactorily on a semipreparative VyDAC Dionex 201SP TM C18 column (201SP510, 10 × 250 mm) running at a flow rate of 2–3 mL/min.

Prior to each run, the columns were washed with 1–2 column volumes of buffer at the maximum eluent concentration associated with each system (SAX: 900 m*M* NaCl/25 m*M* Tris–HCl pH 8.9 or 900 m*M* KCl/100 m*M* NH_4OAc/2% CH_3CN pH 8.0; RP: 100% CH_3CN). The columns were then equilibrated with 1–2 column volumes of buffer at the starting

conditions before the samples were loaded. Unless otherwise specified, all separations were conducted at room temperature (22–24 °C). In some instances, we have found that increasing the temperature to 45–65 °C can sharpen diastereomeric peaks and increase the separation time.

4. Examples of Phosphorothioate Oligonucleotide Separations

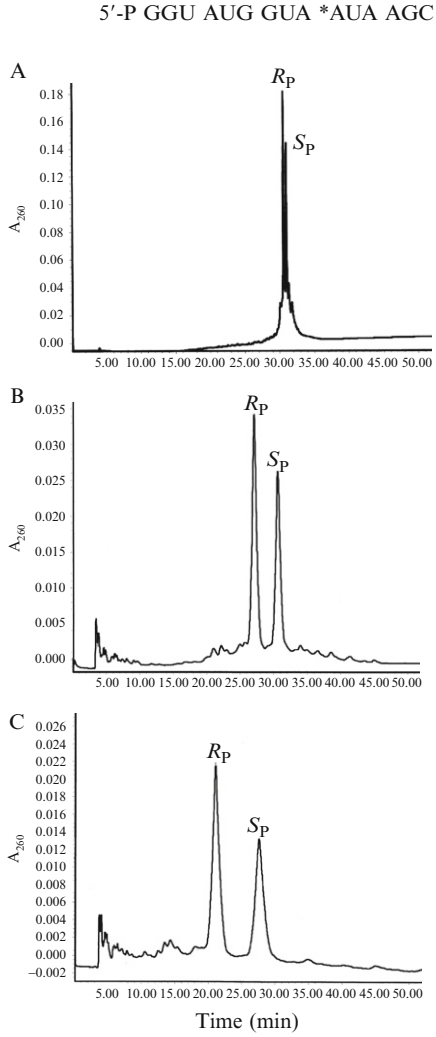
4.1. Solvent system 1: Strong anion exchange

- Solvent A: 0.25 *M* Tris–HCl pH 7.4–8.9
- Solvent B: H_2O
- Solvent C: 1 *M* NaCl

In this system, the percentage of solvent A is kept at 10% so that the background buffer concentration is 25 m*M* Tris–HCl. The gradient is created by gradually increasing the percentage of solvent C (the concentrated salt solution) in the mixture, with water making up the difference. Thus, the salt concentration can range from 0 to 0.9 *M* NaCl.

Figure 14.5 shows the successive optimization of HPLC conditions for the separation of a 25-nt RNA phosphorothioate. The initial gradient is relatively steep (+17.5 m*M* NaCl per minute, Fig. 14.3A) so as to determine the approximate salt concentration at which the diastereomers elute from the column. In most but not all instances, the observation of some separation even with this steep gradient indicates that further separation will be possible as conditions are refined. Following general HPLC principles, the separation time between the peaks should increase as the gradient becomes progressively shallower. In this case, starting at 450 m*M* NaCl and reducing the gradient to +2.5 m*M* NaCl per minute increased the separation time to 3.5 min (Fig. 14.5B). Further refinement of the gradient produced the trace in Fig. 14.5C, whose conditions were deemed suitable for preparative separation of the diastereomers.

Once the separation conditions have been optimized, larger quantities of phosphorothioate oligonucleotide may be loaded and separated. The peaks associated with these preparative runs are considerably broader and more asymmetrical compared to the peaks of the test runs (Fig. 14.6A). In addition to maximizing peak separation time during test runs, setting a baseline absorbance (usually 0.1–0.2 A_{260}) that defines the beginning and end of peak elution during preparative runs can help to avoid cross-contamination. Following preparative separation, excess salt must be removed from the pooled fractions of each phosphorothioate diastereomer. For oligonucleotides at least 10 bases in length, desalting may be accomplished by concentrating the oligonucleotides to dryness using a speedvac, resuspending in a



A: 0.25 *M* tris-HCl pH 8.9
B: H_2O
C: 1 *M* NaCl

Time (min)	%A	%B	%C
0	10	80	10
10	10	80	10
50	10	10	80

0.4 min separation

Time (min)	%A	%B	%C
0	10	45	45
10	10	45	45
50	10	35	55

3.5 min separation

Time (min)	%A	%B	%C
0	10	43	47
10	10	43	47
50	10	40	50

6.5 min separation

Figure 14.5 Separation of phosphorothioate diastereomers of the 25-nt RNA 5′-P GGU AUG GUA *AUA AGC UGA CGG ACA U-3′ (5′-P indicates the presence of a 5′-phosphate group, while * marks the position of the phosphorothioate linkage). A_{260} is the absorbance of the HPLC eluent measured at 260 nm. Conditions: Semi-preparative SAX column with a flow rate of 2 mL/min and a sodium chloride gradient of (A) 17.5 m*M*/min, (B) 2.5 m*M*/min, or (C) 0.75 m*M*/min. These gradients are approximations of the true rate of increase of the sodium chloride concentration, since the HPLC pumps utilized in these separations dispense solvents only in increments of 1%. Note that the axes do not show identical scales.

small volume of water (≤1 mL), and passing over a gel filtration column such as a NAP-10 Sephadex G-25 column (GE Healthcare). Alternatively, the phosphorothioates may be desalted by passage through a Sep-Pak C18

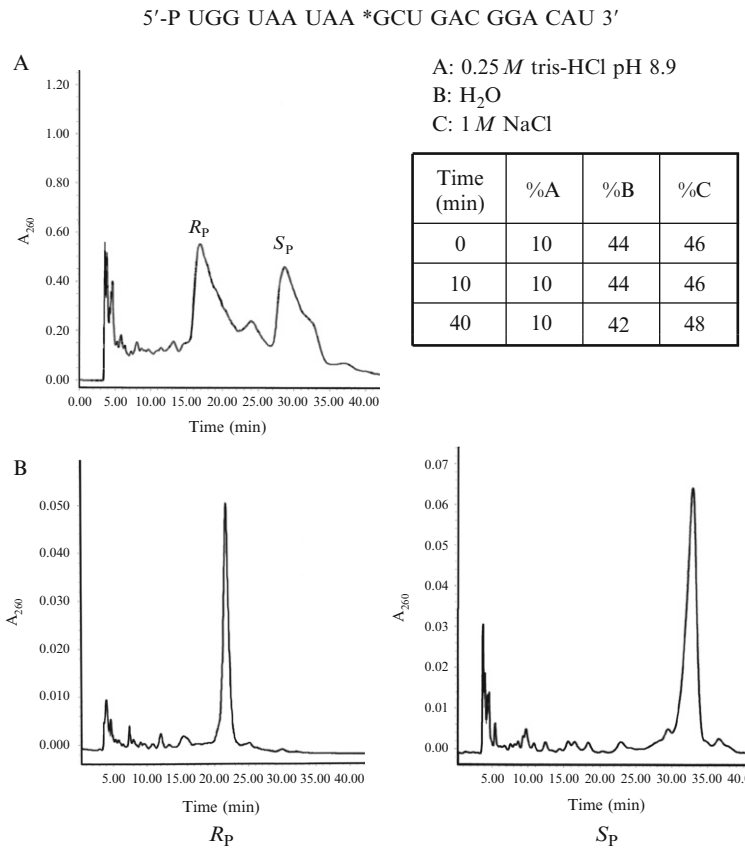
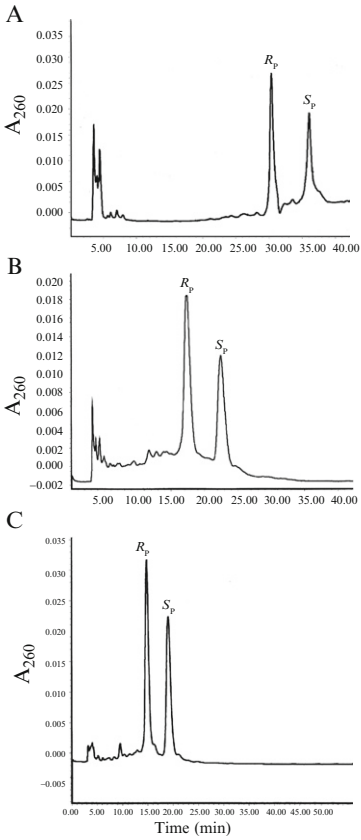


Figure 14.6 **Preparative separation of the 21-nt RNA 5′-P UGG UAA UAA *GCU GAC GGA CAU-3′. Conditions: Semipreparative SAX column with a flow rate of 2 mL/min. (A) HPLC trace showing a representative preparative separation. (B) HPLC traces of individual R_P and S_P diastereomers following separation and desalting.**

cartridge (Waters) or by dialyzing against 2 m*M* sodium citrate pH 6.5 at 4 °C (1:1000 volume differential with three buffer changes at 1–2 h intervals). These latter techniques are not as volume-dependent as gel filtration, and therefore may be more practical for desalting large volumes of eluted material. Oligonucleotides shorter than 10 bases should be desalted either via a Sep-Pak cartridge or by dialyzing, taking care to select dialysis tubing with an appropriate molecular weight cutoff value. After the desalted phosphorothioates have been concentrated, their purity should be assessed by HPLC (Fig. 14.6B).

As Fig. 14.7 shows, the gradients based on this solvent system may be adjusted to separate several different phosphorothioate oligonucleotides of



A: 0.25 *M* tris-HCl pH 8.9
B: H_2O
C: 1 *M* NaCl

5′-P CUU UGA GAU *GGC CUU GCA AAG 3′

Time (min)	%A	%B	%C
0	10	50	40
10	10	50	40
40	10	40	50

5.7 min separation

5′-P AGC CGU UCA GUA CC*A AGU CUC AG 3′

Time (min)	%A	%B	%C
0	10	44	46
10	10	44	46
40	10	42	48

5.1 min separation

5′-P CAA AGG GUA UGG UA*A 3′

Time (min)	%A	%B	%C
0	10	45	45
10	10	45	45
50	10	44	46

4.2 min separation

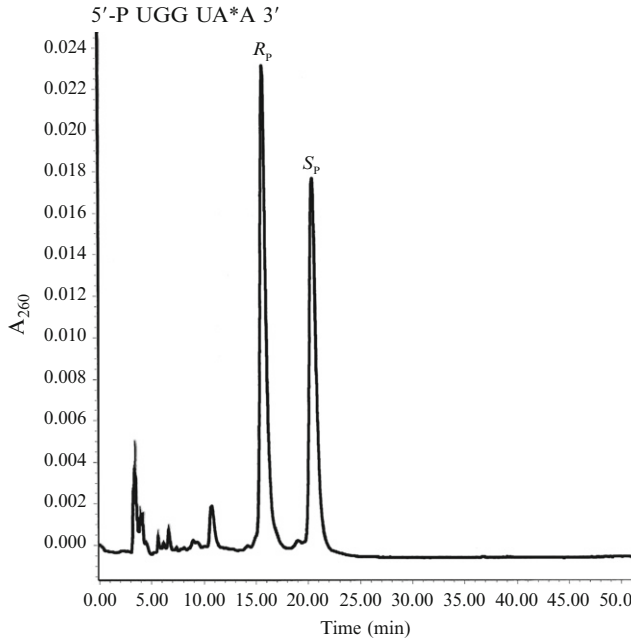
Figure 14.7 Representative phosphorothioate oligonucleotides separated by using SAX HPLC. (A) 21 nt. (B) 23 nt. (C) 15 nt. Conditions: Semipreparative SAX column, flow rate 2 mL/min.

various sequences and lengths. We have used this system to separate phosphorothioates on the order of 15–25 nucleotides, although this range is relatively arbitrary.

4.2. Solvent system 2: Strong anion exchange

- Solvent A: 1 *M* NH_4OAc, 20% CH_3CN, pH 8.0
- Solvent B: H_2O
- Solvent C: 1 *M* KCl

This system may be appropriate for shorter phosphorothioates, up to about 15 nt (Dertinger *et al.*, 2000). Again, solvent A is kept at 10% throughout the run so that the background buffer concentration is 0.1 *M* NH_4OAc, 2% CH_3CN, pH 8.0. Figure 14.8 shows a representative separation for a 6-nt phosphorothioate. Concentration and desalting procedures are the same as for other SAX solvent systems, although NAP-10 columns should not be used for oligonucleotides shorter than 10 bases.



A: 1 *M* NH_4OAc, 20% CH_3CN, pH 8.0

B: H_2O

C: 1 *M* KCl

Time (min)	%A	%B	%C
0	10	70	20
10	10	70	20
50	10	63	27

4.8 min separation

Figure 14.8 Separation of phosphorothioate diastereomers of the 6-nt RNA 5′-PUGG UA*A-3′, using an alternative anion exchange solvent system. Conditions: Semipreparative SAX column, flow rate 2 mL/min.

4.3. Solvent system 3: Reversed phase

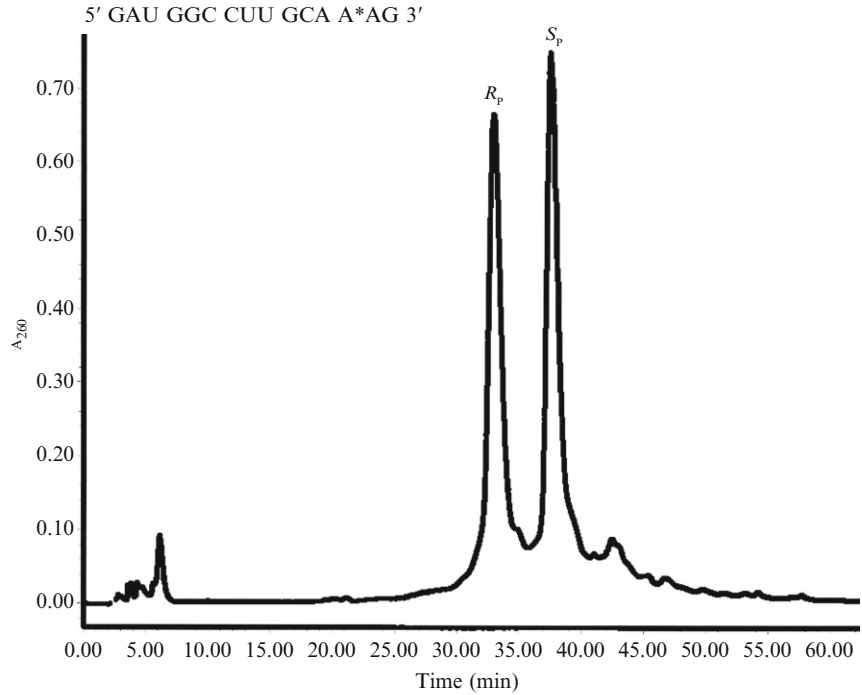
- Solvent A: 0.1 *M* NH_4OAc
- Solvent B: 20% A/80% CH_3CN

This is a widely used RP system that appears in the work of Slim and Gait (1991) and is suitable for separating phosphorothioates of many different lengths (Table 14.1). Figure 14.9 shows the RP preparative separation of a 15-nt phosphorothioate on an analytical C18 column. In this particular separation, elevating the temperature of the column to 45 °C improved the resolution of the diastereomers. Phosphorothioates separated by RP HPLC are ready to use following speedvac concentration and resuspension in water or buffer, and do not require a separate desalting procedure.

4.4. Using complementary DNA oligonucleotides to improve RNA phosphorothioate separation

In the event that suitable resolution of diastereomers cannot be achieved, we present an additional method that has yielded favorable results for certain intractable phosphorothioates. In this approach, first suggested by Daniel Herschlag and Alexander Kravchuk of Stanford University, the RNA phosphorothioate of interest is hybridized to a fully complementary DNA oligonucleotide prior to loading onto the HPLC (Kravchuk and Herschlag, unpublished results). The DNA and RNA oligonucleotides are combined such that the amount of complementary DNA strand equals or exceeds the amount of phosphorothioate RNA (for test runs, 500–1000 pmol of phosphorothioate oligonucleotide provide a satisfactory UV signal). One-tenth volume of TEN_{150} buffer (10 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 150 m*M* NaCl) and water are added to a final buffer concentration of 1 m*M* Tris–HCl pH 7.5, 0.1 m*M* EDTA, and 15 m*M* NaCl (smaller volumes are preferable, on the order of 20–50 μL). The mixture is heated at 90 °C for 4 min, and then incubated at 37 °C for 1 h before loading onto the HPLC.

Figure 14.10 shows the SAX HPLC traces associated with a 15-nt RNA phosphorothioate both in the absence and in the presence of a complementary DNA oligonucleotide. Using the same gradient, the complementary DNA improves the separation of the phosphorothioate diastereomers dramatically. The peaks may then be collected, concentrated, and desalted in the same manner as described above for phosphorothioates separated via SAX HPLC. Following desalting, however, the complementary DNA is removed by treatment with RQ1 RNase-free DNAse (Promega) according to the manufacturer's instructions.



A: 0.1*M* NH_4OAc
B: 20% A/80% CH_3CN
45 °C

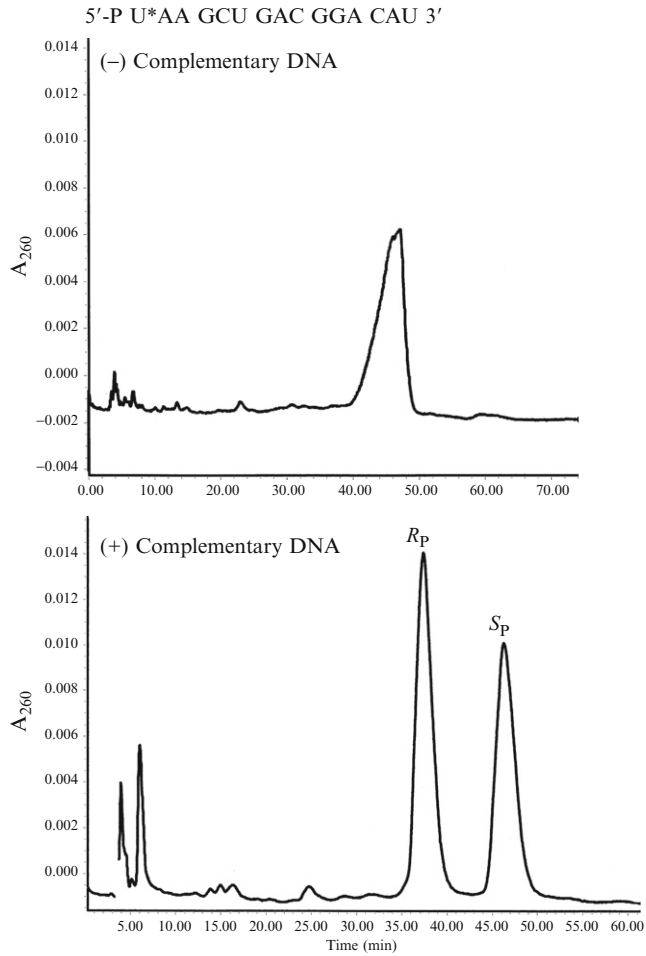
Time (min)	%A	%B
0	97	3
10	97	3
90	90	10

4.6 min separation

Figure 14.9 **Preparative reversed phase separation of diastereomers of the 15-nt RNA 5′-GAU GGC CUU GCA A*AG-3′. Conditions: Analytical RP column, flow rate 1 mL/min, 45 °C.**

4.5. Summary and Conclusion

Separation of oligonucleotide phosphorothioate diastereomers by HPLC is a trial-and-error process affected by a multitude of variables that no single set of conditions can control. We have therefore presented several different strategies that may be attempted should any one approach fail to resolve the



A: 0.25 *M* tris-HCl pH 8.9
B: H_2O
C: 1 *M* NaCl

Time (min)	%A	%B	%C
0	10	49	41
10	10	49	41
70	10	47	43

Figure 14.10 Separation of phosphorothioate diastereomers of the 15-nt RNA 5′-P U*AA GCU GAC GGA CAU-3′. Both HPLC traces use the indicated gradient. Under these conditions, the diastereomers elute as a single asymmetric peak (top panel). However, the resolution improves considerably when the phosphorothioate is hybridized to a fully complementary DNA oligonucleotide prior to loading on the HPLC (bottom panel). While not apparent on this trace, the much shorter retention time of excess complementary DNA usually suffices to distinguish it from phosphorothioate RNA. Conditions: Semipreparative SAX column, flow rate 2 mL/min.

diastereomers. Our list is by no means comprehensive, but may be a convenient starting point from which optimal separation conditions may be found.

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