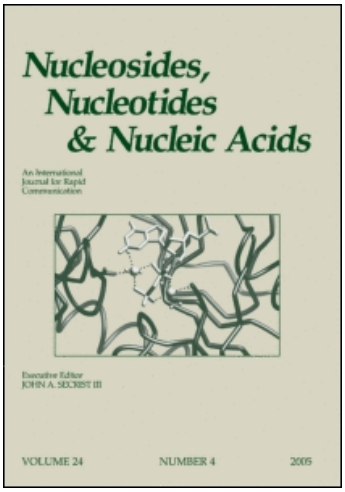

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Thin Layer Chromatography of Radiolabeled Oligonucleotide Analogues: A Rapid and Sensitive Purity Assay

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THIN LAYER CHROMATOGRAPHY OF RADIOLABELED OLIGONUCLEOTIDE ANALOGUES: A RAPID AND SENSITIVE PURITY ASSAY

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Abstract Oligonucleotide analogues are being developed for use in cell culture, animals and for therapy. Prior to use it is important to have an indication of the oligomers' purity. Thin layer chromatography (TLC) has been applied to analyze phosphoromonothioate and phosphorodithioate oligonucleotides radiolabeled with either ^{32}P or ^{14}C. TLC coupled with radioactivity has been compared to Polyacrylamide Gel Electrophoresis (PAGE) and High Pressure Liquid Chromatography (HPLC). TLC is a rapid and sensitive alternative to these methods and is particularly suited for chemically modified oligonucleotides.

Traditionally oligonucleotide analysis has been accomplished either by reversed-phase HPLC or by enzymatic radiolabeling of an oligomers' 5' end with polynucleotide kinase and $\gamma^{32}P$-ATP followed by PAGE. Capillary Gel Electrophoresis (CGE), a more recent method, while rapid, sensitive and automated is not yet generally available. Backbone-substituted oligomers such as phosphorothioates[1,2,3], methylphosphonates[4] and phosphorodithioates[5] are becoming increasingly used perhaps because of adaptability to solid-support synthesis, nuclease-resistance and improved pharmacological characteristics. However these and other chemically modified oligomers can be difficult to label by enzymatic means and may not be amenable to electrophoretic methods of analysis.

Recently we reported on chemical radiolabeling of methylphosphonate and phosphorothioate oligomer analogues[6] with ^{14}C. The oligomer attains ^{14}C via reaction with ^{14}C-Formaldehyde(American Radiolabeled Chemicals), followed by reduction with sodium cyanoborohydride(Fluka). The ^{14}C labeled oligomers could be detected by TLC. Briefly, this is a "one-pot" procedure which involves stirring the oligonucleotide in the presence of ^{14}C-CH_2O for 1 hr, with subsequent addition of 10 mM CH_3BNNa and reaction for 8 hrs. The [^{14}C]-Oligomer is then precipitated with excess alcohol. Because the reaction results in radiolabeling the nucleotide base, it should be adaptable to many types of oligomer analogues. For example, a phosphorodithioate oligomer was radiolabeled using this procedure and assayed by TLC as shown in Figure 1. The

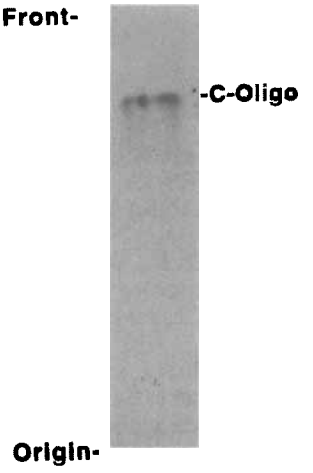

FIG. 1 Analysis of ^{14}C radiolabeled phosphorodithioate d-TACGCCAACAGCTCC (5'-3') "C-Oligo" by TLC[7]

resulting [^{14}C]-TACGCCAACAGCTCC (5'-3') phosphorodithioate oligomer "C-Oligo" gave a single spot (Rf=0.78).

TLC involves spotting several microliters of the radiolabeled oligomer dissolved in 1:4 acetonitrile:water onto a TLC plate(United States Biochemical "Surecheck"). The oligomer is then eluted for 1 hr (in United States Biochemical "Elution Mixture"). After drying the plate for several minutes an autoradiographic exposure is obtained on Kodak X-OMAT AR film.

TLC analysis is also compatible with ^{32}P labeling. To demonstrate, a phosphoromonothioate oligomer of the same sequence used previously was labeled with ^{32}P using polynucleotide kinase and $\gamma^{32}P$-ATP by standard means[8]. The resulting [^{32}P]-TACGCCAACAGCTCC (5'-3') "P-Oligo" was assayed by TLC (Figure 2a). As a comparison, PAGE(Figure 2b) and HPLC(Figure 2c) was also performed on this oligomer. All three methods gave a single major band or peak indicating high purity.

The advantages of TLC versus PAGE or HPLC are time of analysis and sensitivity as summarized in Table 1. Thus the TLC method (not including time for autoradiographic visualization) requires 1 hr in comparison to 4-5 hrs for PAGE. For sensitivity, using TLC coupled to radioactivity affords the detection of 0.01 pmol of oligonucleotide versus 1 pmol by HPLC.

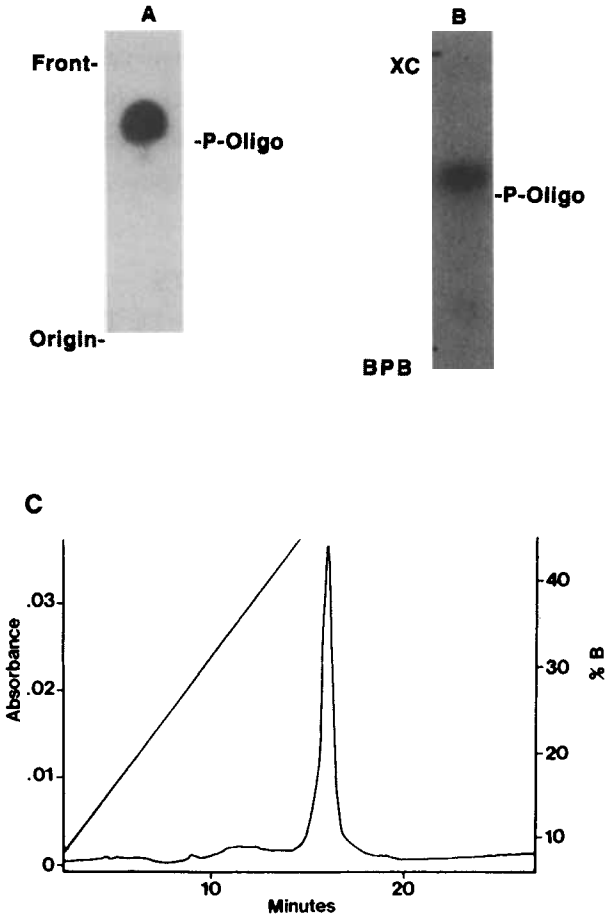

FIG. 2 Analysis of phosphorothioate [^{32}P]-p-d-TACGCCAACAGCTCC (5'-3') "P-Oligo" by TLC(a), PAGE(b) and HPLC(c) of the unphosphorylated form[7]

TABLE 1 Comparison of Analytical Techniques for Assessing Oligonucleotide Purity

Analysis Type	Sensitivity(pmol)	Estimated Time(hrs)
TLC w/o radioactivity[9]	10	1
w/ radioactivity	0.01-0.1	1-2
PAGE w/ radioactivity	0.01-0.1	4-5
HPLC w/o radioactivity	1	<1

In summary, TLC analysis of radiolabeled oligonucleotide analogues is a simple rapid and sensitive technique that can be used in laboratories lacking sophisticated analytical equipment. Used in conjunction with base-specific C-14 radiolabeling as described, the TLC method provides an alternative for assessing the purity of oligonucleotide analogues which are difficult to label, and may not be analyzed by other means. TLC may thus be useful to quickly check the purity of oligonucleotide analogues.

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7. [^{14}C]-TACGCCAACAGCTCC (5'-3') phosphorodithioate "C-Oligo"or [^{32}P]-p-TACGCCAACAGCTCC (5'-3') phosphoromonothioate "P-Oligo" was obtained as described in the text or in references 6 and 8 respectively. 10^5 cpm was used for either "P-Oligo" or "C-Oligo". Electrophoresis was carried out on a 15% polyacrylamide/7M urea gel within an Hoefer unit at 500 volts for 2 hrs in 2000 mls of 1X TBE (Gibco-BRL) in the presence of tracking dyes' bromophenol blue(BPB) and xylene cyanol(XC). For HPLC, 0.1 OD_{260} units of phosphorothioate [HO]-d-TACGCCAACAGCTCC$_{OH}$ (5'-

3') was dissolved in 100 µl 2% acetonitrile/20mM triethylaminoacetate, pH 6 and chromatographed on a Waters µBondapack C-18 HPLC column with detection at 260 nm and sensitivity of 0.08 AUFS. A gradient of 0-40% acetonitrile buffered with 20mM triethylaminoacetate in 30 minutes was used. Under these conditions the flow rate was 1ml/min, and pressure constant at 90-100 bars.

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9. Sensitivity is shown as the lower limit of detection for oligonucleotides on TLC, PAGE or HPLC. In a dot-blot of oligonucleotide on TLC a $\geq$10 pmoles could be detected upon visualization using a hand-held ultra-violet lamp. 10^5 cpm of ^{14}C radiolabeled compound is sufficient for detection by autoradiography (1-hour exposure). Sensitivity is enhanced greatly (0.01-0.1 pmol) using ^{32}P-oligomer because of high specific activity (3000µCi/nmol). For HPLC the detection limit is 0.0001-0.001 OD_{260} units (approximately 1-10 pmoles) with UV detection set at high sensitivity (0.001 AUFS). Time required to perform each type of analysis was determined empirically.

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