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Note

Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography

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There is an increasingly widespread use of synthetic oligodeoxynucleotides, more recently in the field of selective gene regulation by "antisense oligonucleotides"[1], with consequent therapeutic implications. The selection of phosphate backbone modified analogues of oligonucleotides, such as methylphosphonate[2–4], phosphorothioate[5–8] and phosphoramidate[6,7] provides nuclease resistance and has greatly enhanced the possibility of their use *in vivo*. Since the purity and chemical identity of

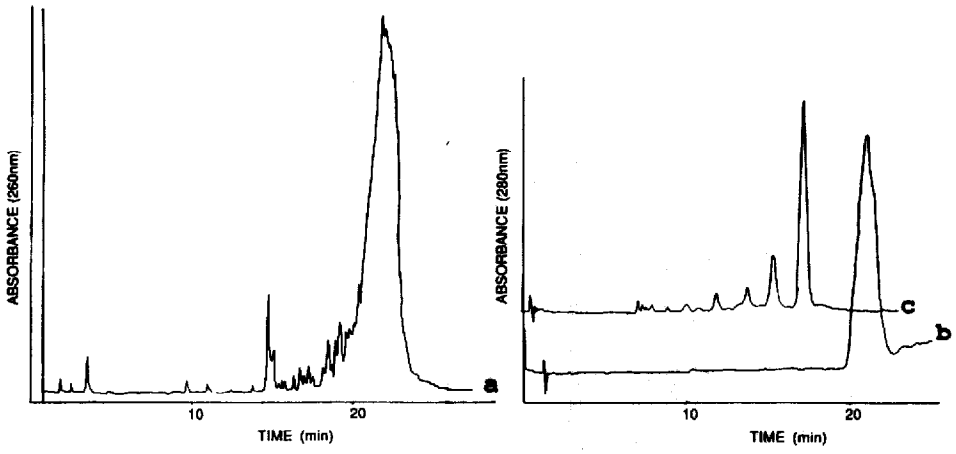

Fig. 1. HPLC traces of (a) crude phosphorothioate octamer, ACTGATGC on reversed phase, (b) purified phosphorothioate octamer on reversed phase and (c) purified phosphorothioate octamer on Partisphere SAX. Conditions: HPLC system consists of Waters 600E system controller, Waters Lambda Max Model 481 detector, Waters 745 data module, reversed-phase column Novapak C_{18} with RCM 100, ion-exchange column Partisphere SAX. For reversed-phase HPLC the buffers were 0.1 *M* ammonium acetate (pH unadjusted) containing (A) 0% acetonitrile and (B) 80% acetonitrile. Gradient was 0% B 2 min, 0–60% B 35 min, flow-rate, 1.5 ml min^{-1}. For ion-exchange HPLC the buffers were prepared from a stock solution of 1 *M* KH_2PO_4 adjusted to pH 6.3 with KOH to give (A) 1 m*M* and (B) 0.4 *M* both containing 60% formamide, flow-rate, 2 ml min^{-1}, gradient was 0% B 2 min, 0–70% B 35 min.

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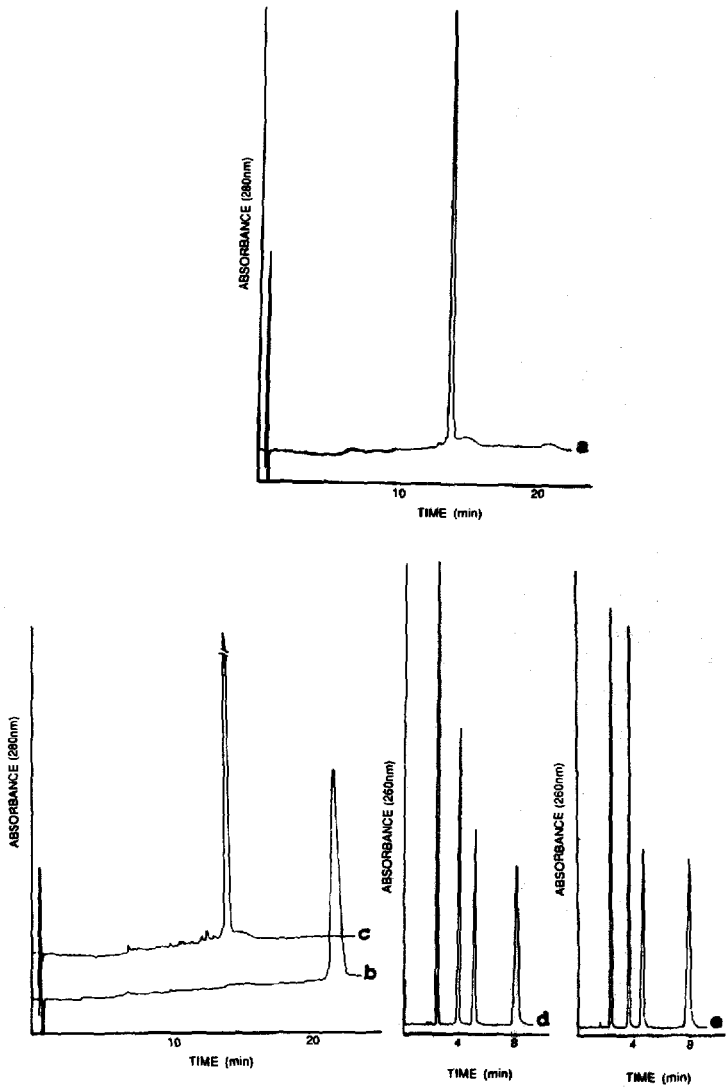

Fig. 2. HPLC traces of (a) purified phosphodiester octamer, ACTGATGC on Partisphere SAX, (b) purified phosphorothioate octamer, ACTGATGC on Partisphere SAX, (c) phosphorothioate octamer oxidised with sodium metaperiodate, (d) and (e) base composition analysis of phosphodiester octamer (authentic) and phosphodiester octamer obtained after oxidation of phosphorothioate octamer, respectively, after digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. Conditions: HPLC system, buffers and columns were same as in the legend to Fig. 1. Gradient for HPLC (a), (b) and (c) was 0% B 2 min, 0–60% B 40 min, and gradient for HPLC (d) and (e), 5% A in 95% B isocratic.

a particular oligonucleotide is crucial to many applications, the ability to characterize synthetic oligonucleotide analogues on a routine basis is important. However, the analytical study of phosphorothioates has not been reported heretofore.

Phosphorothioate analogue of oligonucleotides can be assembled using either methoxyphosphoramidite[9] or H-phosphate chemistry[6,10]. These are diastereomeric

mixtures due to chirality at phosphorus. As a result, although they migrate like corresponding phosphodiester oligonucleotides on gel electrophoresis, on high-performance liquid chromatography (HPLC) the phosphorothioate oligonucleotides give broder peaks than phosphodiester oligonucleotides and also run slower because of their increased hydrophobicity. Fig. 1a shows the elution of a crude octamer, ACTGATGC, from a reversed-phase C_{18} column and, in Fig. 1b the elution of the purified material. When this is then chromatographed on an ion-exchange column shorter oligomers present are separated, as shown in Fig. 1c, allowing the isolation of the pure octamer. Thus, reversed-phase chromatography in itself is insufficient to purify phosphorothioate oligonucleotides. Phosphorothioate analogues are somewhat resistant to nucleases[11], and for the analysis of the base composition (and if necessary the sequence) conversion to the phosphodiester oligonucleotide is required. We have recently found that phosphorothioate oligonucleotides are rapidly and cleanly oxidised to the corresponding phosphodiester oligonucleotides by sodium meta-periodate in water. Iodine-bicarbonate oxidation is fast and equally effective and has been used for desulphurising oligomers containing one or two phosphorothioate internucleotide linkages[12,13]. When the purified phosphorothioate octamer (Fig. 2b) is oxidised it is converted to the phosphodiester octamer (Fig. 2c) identical in elution time to an authentic sample of the latter (Fig. 2a). The oxidised phosphorothioate octamer when digested with snake venom phosphodiesterase and bacterial alkaline phosphatase[14], gave a nucleoside mixture separable by HPLC (Fig. 2d and e) identical with that derived from the authentic phosphodiester oligomer[a], giving the correct base composition (A–G–T–C, 1:0.98:1:0.97). This procedure has been applied successfully to a number of other phosphorothioate oligomers including several 20-mers.

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[a] Assembly of the octamer was carried out using H-phosphonate chemistry. The intermediate CPG-bound octamer H-phosphonate before oxidation was divided into two portions. One portion oxidised with 2% iodine in pyridine–water (98:2) and used as an authentic sample. The other portion oxidised with 5% sulphur in triethylamine–pyridine–carbon disulfide (1:10:10) and used for oxidation with sodium metaperiodate.

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