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HIGH-SPEED PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SYNTHETIC OLIGONUCLEOTIDES

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SUMMARY

A rapid method is described for the purification and analysis of synthetic oligonucleotides, based on reversed-phase high-performance liquid chromatography. Volatile buffers and a short column (40 mm × 4.6 mm) packed with Nucleosil 300-5 C_4 were employed. Monitoring the column effluent with an UV detector provides an excellent means of controlling product quality. The method is suitable for the purification of crude synthesis products, as well as for desalting and removing gel contaminants from oligonucleotides eluted from polyacrylamide gels. The total time required per sample is less than 25 min.

INTRODUCTION

Synthetic oligonucleotides have many important uses in molecular biology, *e.g.*, DNA sequencing, linker technology, gene synthesis and gene-bank screening. The high productivity of synthesizers often means that product purification and quality control constitute a bottle-neck. It is therefore desirable to have a rapid means of removing by-products and failed sequences from crude synthesis products. Ion-exchange chromatography may be used[1,2], but the samples have subsequently to be desalted. Reversed-phase high-performance liquid chromatography (HPLC) is an alternative technique[2–5]. The method presented here, based on reversed-phase HPLC, uses volatile buffers and requires only 25 min per sample, including column equilibration. It is possible to purify crude synthesis products as well as quickly and easily to desalt and remove gel contaminants from oligonucleotides eluted from polyacrylamide gels. The latter is of particular importance, since gel contaminants often interfere with enzymatic reactions. In addition, UV detection is an excellent method of controlling the purity of products and quantifying yields. Therefore, the method presented here represents a useful tool for the analytical control as well as the preparative purification of synthetic oligonucleotides.

MATERIALS AND METHODS

Oligonucleotide synthesis

The oligonucleotides were synthesised in an automatic synthesizer constructed in our laboratory[6] and using phosphotriester chemistry[7].

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HPLC equipment

The HPLC equipment consisted of two Model 100a HPLC pumps (Beckman, Palo Alto, CA, U.S.A.), an HPLC controller constructed in our workshop, an Uvidec 100-II variable wavelength UV detector (Jasco, Tokyo, Japan), a BD40 chart recorder (Kipp & Zonen, Delft, The Netherlands), a Beckman HPLC injection valve and gradient mixing chamber.

Chemicals

Acetonitrile was of HPLC grade from Baker (Deventer, The Netherlands), triethylamine was of synthesis grade from Merck (Darmstadt, F.R.G.) and acetic acid of analytical grade from Merck.

HPLC conditions

The column filled with Nucleosil 300-5 C_4 (40 mm × 4.6 mm) was packed by Bischoff (Leonberg, F.R.G.). Eluent A was 0.1 *M* triethylamine titrated to pH 6.5 with glacial acetic acid. Eluent B was 100% acetonitrile. The flow-rate was 0.5 ml/min. Oligonucleotides with the trityl protecting group attached were separated as follows: isocratic at 20% B for 2 min; linear gradient from 20 to 40% B over 10 min; isocratic for 2 min; linear gradient from 40 to 20% B in 2 min; equilibration at 20% B for 10 min. After cleavage of the trityl protecting group a different elution scheme was used: linear gradient from 5 to 25% B over 10 min; isocratic for 2 min; linear gradient from 25 to 5% B over 2 min; equilibration at 5% B for 10 min. For preparative chromatography, in order to avoid out-of-range detector signals, the absorption of the column effluent was monitored at 290 nm and not at the absorption maximum of DNA, 260 nm. The detector was also equipped with a preparative cuvette of pathlength 1 mm. A cuvette with a pathlength of 10 mm was used for analytical chromatography.

RESULTS AND DISCUSSION

Figs. 1–4 show the results of the purification of a 27-mer synthetic oligonucleotide. The preparative purification of the oligomer before removal of the trityl protecting group is illustrated in Fig. 1. The product peak represents 25 A_{260} units of crude DNA. The chromatogram clearly shows that the peak of the synthesis product is well separated from hydroxylated failure sequences (peak A), benzamide and other by-products produced during synthesis and deprotection (peak B). The gradient system described above was tested with oligonucleotides up to 70 bases in length.

A major problem in the preparative purification of oligonucleotides is that high peak concentrations overload the UV detector, resulting in a poorly resolved chromatogram. To overcome this difficulty, the detector was fitted with a preparative cuvette of pathlength 1 mm and the absorption was measured at 290 nm. By this means it is still possible, even with a very high sample load, to produce a clear chromatogram.

Fig. 2 shows an analytical chromatogram of the same oligomer after the first purification step (Fig. 1) and subsequent removal of the trityl protection group. In this case an analytical cuvette having a pathlength of 10 mm and a wavelength of 260 nm were employed. The product was shown to be approximately 90% pure, by

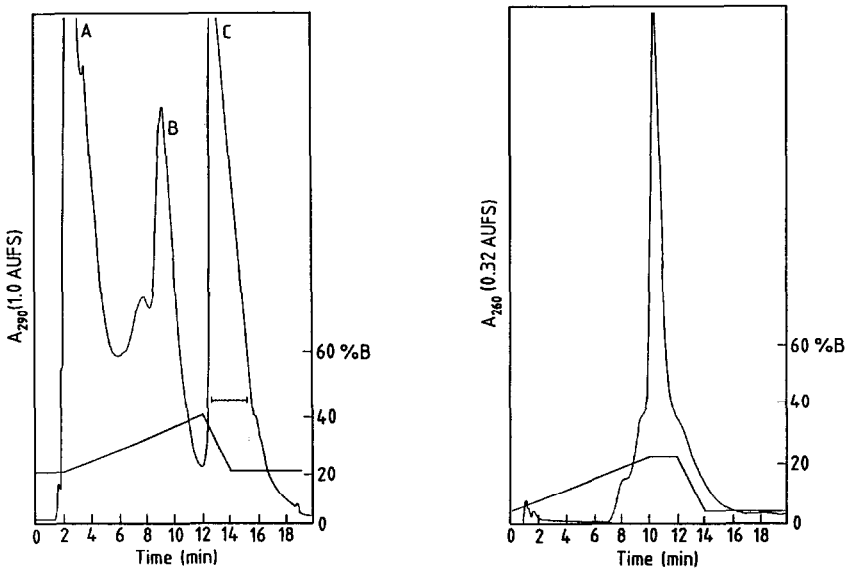
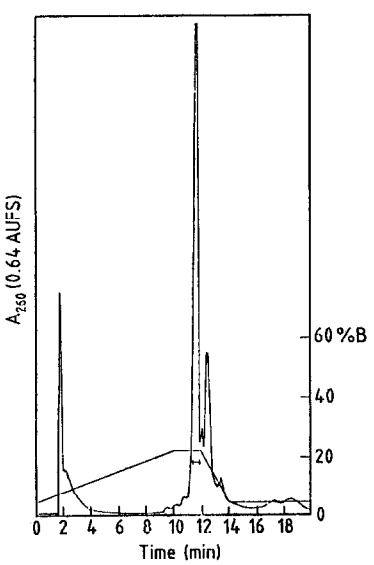


Fig. 1. Preparative purification of a 27-mer before removal of the trityl protection group. The product peak represents *ca.* 25 A_{260} units of DNA. The detector was fitted with a preparative cuvette of pathlength 1 mm and the absorption was measured at 290 nm. The product peak was collected, as indicated.

Fig. 2. Analytical chromatogram of the 27-mer after the first prification step (Fig. 1) and subsequent removal of the trityl group. The detector was fitted with an analytical cuvette of pathlength of 10 mm, and the absorption was measured at 260 nm.

polyacrylamide gel electrophoresis. This is adequate for some applications. However, where it is not sufficient, an additional purification on a denaturing polyacrylamide gel containing urea offers a high-resolution technique, capable of handling a number of samples simultaneously. A disadvantage of gel purifification is the need to desalt and remove gel contaminants in the samples after elution. This may be achieved by ethanol precipiation, or by batchwise extraction on reversed-phase cartridges. Neither of these methods provides a direct means of assessing the yield and/or contamination resulting from the elution procedure. Fig. 3 clearly demonstrates the advantage of the method described above. The UV trace shows that the product obtained from the gel was still contaminated, but the contaminants can easily be eliminated, by fractionating the column effluent. The result of such a fractionation is shown in Fig. 4.

Further, the volatile eluents[2–4] used can be removed under vacuum, without recourse to dialysis and/or ethanol precipitation. The use of different detector cuvettes and wavelength settings away from the absorption maximum of DNA permits maximum flexibility, the same column serving for both preparative and sensitive analytical chromatography. The UV detector provides an adequate means of controlling sample quality, especially after gel purification. Oligonucleotides purified in this way have been shown, in our laboratory, to be good substrates for polynucleotide kinase and to give high ligation yields during gene synthesis (results not shown).



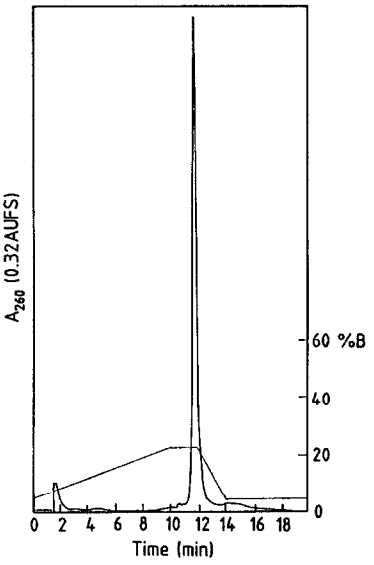


Fig. 3. Chromatogram of the prepurified 27-mer, after further purification on a polyacrylamide gel. Note the UV-absorbing contaminants. The product peak was collected as indicated.

Fig. 4. Chromatogram of the 27-mer, after fractionation to remove the UV-absorbing contaminants visible in Fig. 3.

In conclusion the reversed-phase HPLC purification and analysis of synthetic oligonucleotides described here is a useful tool for obtaining pure synthetic oligonucleotides and monitoring their quality.

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