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Is It Essential to Use Anhydrous Acetonitrile in the Manufacture of Phosphorothioate Oligonucleotides?

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Abstract:

It is demonstrated that acetonitrile containing up to at least 200 ppm water can be used as the wash solvent and to prepare the capping and sulfur transfer reagent solutions used in large-scale solid-phase phosphorothioate oligonucleotide synthesis, thus replacing most of the expensive anhydrous acetonitrile previously used. No compromise in yield or product quality is observed, compared to a control synthesis performed using anhydrous acetonitrile throughout.

The specific binding of synthetic oligonucleotides to mRNA or pre-mRNA sequences through Watson−Crick base pairing can result in highly selective inhibition of gene expression, which is the principal of antisense drug therapy.[1−7] In 1998, the world's first antisense drug, Vitravene (fomivirsen sodium), was approved by the Food and Drug Administration for the treatment of CMV retinitis in AIDS patients.[8] In common with other first and second generation antisense agents, Vitravene is a phosphorothioate oligonucleotide analogue wherein a nonbridging oxygen atom of each internucleotide linkage is replaced by sulfur. A large number of additional antisense drugs are currently in clinical trials.[8] These trials have fueled the need for production of large quantities of synthetic phosphorothioate oligonucleotides. This in turn has stimulated considerable effort towards developing a cost efficient synthesis process.[9,10] As part of our ongoing work[11−13] directed to minimizing the cost of oligonucleotide chemical synthesis, we now wish to report results regarding the necessity of anhydrous acetonitrile (MeCN) use in oligonucleotide chain assembly.

Phosphoramidite coupling has become the chemistry of choice for small- and large-scale synthesis of oligonucleotides. Currently, antisense phosphorothioate oligonucleotides of 20-mer length are routinely synthesized on the Pharmacia OligoProcess DNA/RNA synthesizer at 150 mmol scale using only a 1.75 molar excess of phosphoramidite per coupling.[14] Although we have made considerable progress in reducing the cost of oligonucleotide synthesis, there remain areas for further improvement. One such area concerns the consumption of anhydrous MeCN, which, in addition to being the solvent used to prepare phosphoramidite, tetrazole, capping, and sulfur transfer reagent solutions, is used in large quantities as the process "wash solvent" (Scheme 1) between the steps which constitute a synthesis cycle.

A single 150 mmol synthesis of a phosphorothioate oligonucleotide 20 bases long consumes around 1000 L of MeCN, approximately 90% of which is used in the washing steps. At present, only MeCN containing less than 10 ppm ("anhydrous") of water is used, a characteristic reflected in its rather high price. We reasoned that the process cost of MeCN could be reduced substantially if the specifications regarding water content were made less stringent.[15] We therefore investigated the impact of MeCN water content on the synthesis of phosphorothioate oligonucleotides.

Experimental Section

A 20-mer phosphorothioate oligonucleotide [ISIS 2302; d(GCCCAAGCTGGCATCCGTCA)] was chosen as an example. All syntheses were performed on a Pharmacia OligoPilot II DNA/RNA synthesizer using β-cyanoethyl phosphoramidite synthons (1.5 equiv/coupling, 0.2 M in MeCN). 1*H*-tetrazole (0.45 M in MeCN) was used as activator and phenylacetyl disulfide (PADS) (0.4 M in 3-picoline−MeCN, 1:1 v/v) as sulfur transfer reagent.[11] Capping reagents were made to the recommended Pharmacia

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(8) Vitravene is currently undergoing regulatory review in Europe; ISIS 2302, an inhibitor of ICAM-1, is in a pivotal quality trial for Crohn's disease, Phase II clinical trials for renal transplant rejection, ulcerative colitis, psoriasis, and asthma; ISIS 3521 and ISIS 5132 are in Phase II trials as anticancer agents.
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(14) Large scale Pharmacia DNA/RNA OligoProcess I synthesizer (Pharmacia, Sweden) allows complete synthesis of a 20-mer phosphorothioate oligodeoxyribonucleotide up to 0.2 mole scale in less than 10 h.
(15) MeCN containing up to 30 ppm water can be purchased for about half the price of the anhydrous grade.

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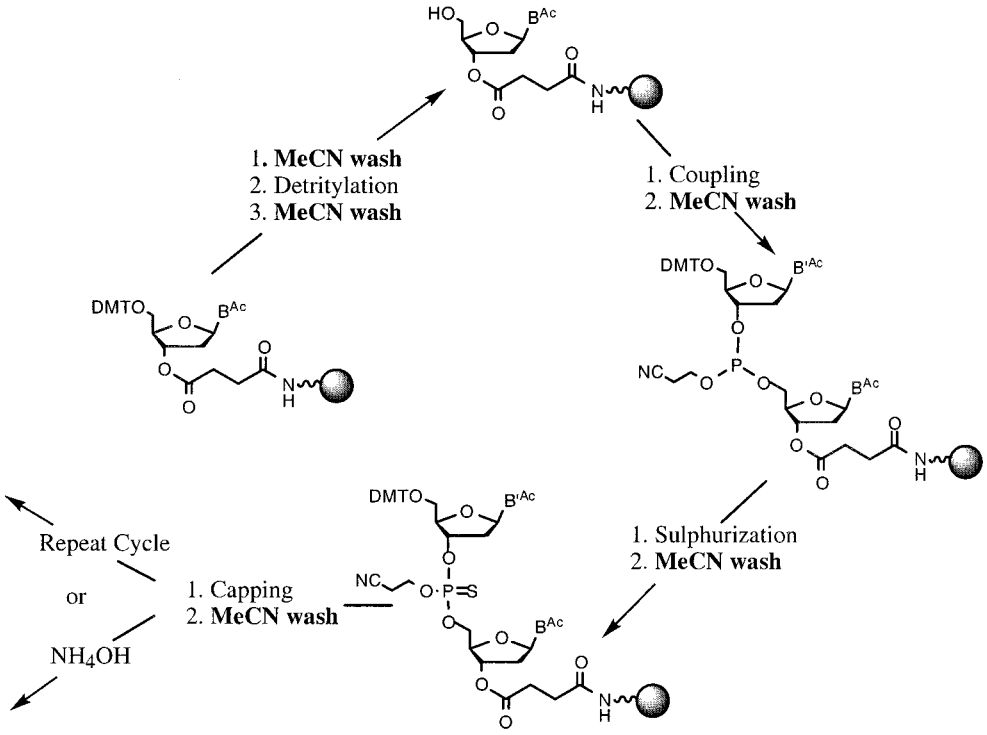
Scheme 1



recipe: Cap A; *N*-methylimidazole-MeCN (1:4 v/v), Cap B; acetic anhydride−pyridine−MeCN (2:3:5, v/v/v). Pharmacia HL 30 Primer Support was used in all experiments. Amidite and tetrazole solutions were prepared using anhydrous MeCN (∼10 ppm) and were dried further by addition of activated 4 Å molecular sieves (∼50 g/l). MeCN of varying water content was prepared by adding known volumes of purified water to anhydrous MeCN, and the final water content of each solvent mixture was estimated by Karl Fischer titration. These solvent mixtures were used both as the wash solvent and to prepare the capping and PADS solutions. At the end of each synthesis, trityl-on support bound oligo was treated with 30% aqueous ammonium hydroxide solution for 16 h at 55 °C to effect release from the support and base and phosphate deprotection. Yield (expressed in mg of oligonucleotide/μmol of support),[16] ^{31}P NMR and capillary gel electrophoresis (CGE) data were collected for each synthesis. In addition, a portion of the crude material obtained from each synthesis was purified by reversed phase HPLC and the purified material examined by CGE.

Results and Discussion

From the early days of phosphoramidite chemistry[17] it was deemed no more than common sense that MeCN used to dissolve both amidites and activator would have to be as dry as possible. At minimum quantity of amidite being used, using less than completely anhydrous solutions of activator and phosphoramidite would simply lead to the hydrolysis of valuable amidite and, hence, a decrease in overall yield. We felt, though, that the MeCN used for the wash steps and in the preparation of capping and sulfur transfer reagents might be considerably wetter before a detrimental effect on synthesis would be observed. This hypothesis was consistent with several factors. First, in regards to the capping solutions, we felt that any water present when the capping solutions were mixed would be removed by acetic anhydride in Cap B. As the capping solutions are mixed in a v/v ratio, this would represent a reduction of only ∼1.5% in the total acetic anhydride available for capping unreacted hydroxyl groups in the case of MeCN containing 400 ppm water. (Assuming 99% average coupling efficiency the number of equivalents of acetic anhydride per unreacted hydroxyl group using standard capping volumes would fall from 600 to 590). Confidence that the strictly anhydrous MeCN used for washing could be replaced by a wetter grade was based on an understanding of the synthesis cycle and the coupling step in particular. The Pharmacia OligoPilot II (and OligoProcess) synthesizers use a reactor design in which the solid support (polystyrene:dextran) is packed into a vessel resembling a preparative chromatography column. Reagents pass through the column as bands, thus minimizing solvent and reagent usage (this is in contrast to the earlier Milligen and ABI synthesizers in which the solid support was agitated while suspended in solvent). The coupling step in each cycle comprises alternate deliveries of activator and phosphoramidite solutions, the first and last deliveries in an individual coupling step being tetrazole. It was thought that bands of tetrazole bracketing the moisture-sensitive activated phosphoramidite solution would be protected against nonanhydrous MeCN left in the column from the previous washing

(16) Experience has taught us that yields expressed in terms of weight/μmol of support are more reliable than those expressed in terms of optical density/μmol.

(17) Beaucage, S. L., Caruthers, M., H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862.

Table 1. **Combined yield and purity data**

synthesis	H_2O content of MeCN)[a]	yield (DNA/μmol of support)	P═S:P═O[b]	full length[c] (%) crude	pure
2302-1	10	7.5	99.2:0.8	68	90.3
2302-2	200	7.6	99.2:0.8	67	90.1
2302-3	400	6.8	99.2:0.8	68	90.5

[a] Estimated by Karl Fischer titration. [b] Measured by SAX chromatography.[20] [c] Measured by CGE.[21]

step. In addition, the trailing tetrazole band could protect against amidite hydrolysis caused by water present in the MeCN used to push the coupling mixture through the reactor. We were more concerned about our ability to use wetter than normal MeCN as solvent for the sulfur transfer reagent. Although it is generally accepted that the presence of water in most sulfur transfer reagents leads to increased levels of phosphate diester linkages (possibly due to reaction of water with the initially formed phosphonium salt intermediates), we were encouraged to find a single report of the use of pyridinium and trialkylammonium tetrathionates[18] in which it was claimed the presence of water led to no increase in unwanted oxidation products.

In total, three experiments were carried out using MeCN of varying water content. Initial experiments (data not shown) convinced us that we were indeed working very close to the minimum excess of phosphoramidite required.[19] This is an important point as it is clear that by using enough amidite one could in theory use extremely wet solvents and not observe a reduction in synthesis yield. In that scenario one would simply be using activated phosphoramidites as an expensive drying reagent. In all three experiments reported here, amidites and tetrazole were made up in anhydrous MeCN and MeCN of the indicated water content was used for washing and to prepare the capping and sulfur transfer reagents. The overall yield, phosphorothioate content and crude full-length data are presented in Table 1.

Figure 1 shows CGE analyses of both crude and reversed phase HPLC-purified synthetic oligonucleotides 2302-1 and 2302-2. It is evident from Table 1 and the electropherograms shown in Figure 1 that MeCN containing up to at least 200 ppm water can be used in the synthesis of a phosphorothioate oligonucleotide like ISIS 2302 without compromising the yield or quality of oligonucleotide produced. Increasing the water content further to 400 ppm results in a ~11% decrease in overall yield, although the quality of the material produced is unchanged. Particularly interesting is the observation that the presence of water in the sulfur-transfer reagent solution does not lead to an increase in the amount of phosphate diester produced.[22]

(18) Efimov, V. A.; Kalinkina, A. L.; Chakhmakhcheva, O. G.; Hill, T. S.; Jayaraman, K. *Nucleic Acids Res.* **1995**, *23*, 4029−4033.

(19) Experiments carried out with lower excesses of amidite always resulted in a lower yield of product. For example 1.4 equiv of amidite/coupling gave only 6.8 mg of oligo/μmol of support.

(20) Three recent runs of the same sequence performed on the Pharmacia OligoProcess synthesizer (150 mmol scale) gave a P═O content of ~0.1%/linkage for each synthesis. The reasons for this enhanced performance are currently under investigation.

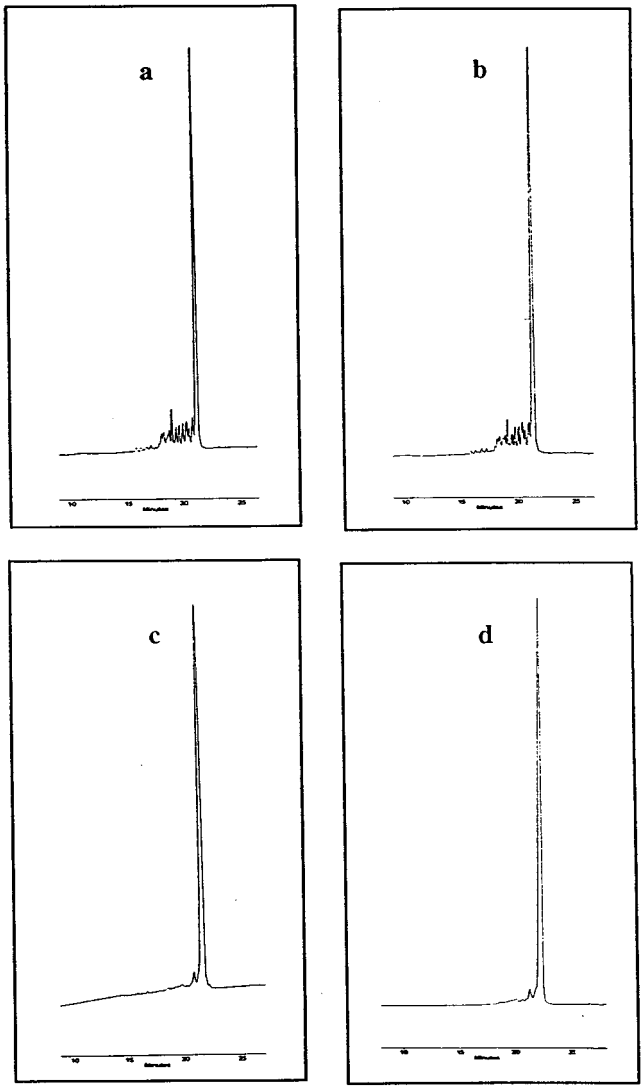


Figure 1. **Capillary gel electropherograms of (a) crude 2302-1, (b) crude 2302-2, (c) HPLC purified 2302-1, and (d) HPLC purified 2302-2.**

Conclusion

In summary, we have demonstrated that it is possible to use MeCN containing up to 200 ppm water for the synthesis of phosphorothioate oligonucleotides. We believe that converting processes from anhydrous MeCN to this wetter grade will add significantly to the striking reductions in large-scale oligonucleotide manufacturing cost that have already been achieved.[9]

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(21) The 90% full length value is typical when the entire trityl-on peak is collected. Much higher purity (~96% full-length) is easily achieved by efficient fractionation of the trityl-on peak. Fractionation of the trityl-on peak results in less than a 5% loss of full length material.

(22) MeCN containing up to 1200 ppm of water, while giving lower yields, gave no increase in phosphate diester content.