CHAPTER 3

Stability of modified antisense ODN under culture conditions

3.1. Introduction

Nuclease degradation may affect the specificity and /or efficiency of ODNs and their stability under physiological conditions is therefore particularly important. Fifteen self-complementary bases were added at the 3'-end of the antisense phosphodiester ODNs as described in the previous chapter in an effort to prevent exonuclease degradation. There are three commonly used and accepted techniques to investigate the stability of ODNs under standard culture conditions. These include capillary electrophoresis (CE), anion exchange HPLC or reversed phase HPLC analyses of culture extracts after pre-incubation with ODN (Giles *et. al*., 2000; Kemp, 1998; Deshmukh *et. al*., 2000). The most appropriate method for analyses was selected after consideration of their principles of operation and general applicability for such studies. The costs associated with the methods and availability of equipment were additional factors taken into consideration when deciding on the most appropriate method to be used for this application.

3.1.1. Capillary Electrophoresis

This method is based on the migration of charged species dissolved or suspended in an electrolyte under the influence of an electric current. Detection is typically performed by UV absorbance. Sample injection occurs by placing one end of the capillary tube into a sample vial followed by the application of an electric field or gas pressure (Kemp, 1998).

There are different types of CE of which capillary zone electrophoresis (CZE) is the most commonly used method to separate different sized ODNs as well as DNA based on the migration of particles determined by the charge/size ratio (Kemp, 1998). Most of these capillaries are typically 30 – 50 µm in diameter and may have a hydrophobic coating that serves to mask the surface charge intrinsic to the capillary tube and thus prevent undesired electro osmotic flow (EOF) (Kemp, 1998). The Hewlett Packard Oligonucleotide Analysis kit (Hewlett Packard) makes use of a PVA (polyvinyl alcohol)-coated capillary tube, which eliminates EOF and suppresses interactions between particles and the inner capillary wall. Alternatively, use is also made of a polymer sieving solution or low-percentage polyacrylamide gel-filled capillaries (Hewlett Packard Oligonucleotide Analysis Kit Information Brochure)

Although capillary electrophoresis has been successfully used for quantification of ODNs, the gel columns utilized in CE are subject to interference by biological media (Bansal *et al*, 2001). Extensive treatment (clean-up) of ODNs incubated in biological samples, including precipitation or anion exchange HPLC followed by reversed phase HPLC, is required prior to CE analysis. The reason for this is that protein and salt interfere with the amount of sample that can be applied to gel-filled capillaries by electrokinetic injection (Leeds *et. al*., 1996). The implication of the above is that determination of ODNs from biological samples by CE is a costly and time-consuming procedure (Bansal *et. al*., 2001; Leeds *et. al*., 1996).

3.1.2. High Performance Liquid Chromatography (HPLC)

Anion exchange or reversed phased HPLC are routinely employed for both the purification and analysis of oligonucleotides.

3.1.2.1. Anion Exchange HPLC (AE HPLC)

Separation by AE HPLC is based on the electrostatic interaction between the negatively charged phosphate groups of the phosphodiester ODN or DNA backbone and the positively charged anion exchange matrix. The ODNs are desorbed from the column by a gradual increase in the ionic strength of the eluting buffer. Due to the relationship between the length and net charge, shorter ODNs elute earlier from these columns (Borque and Cohen, 1993, 1993; Deshmukh *et. al*., 2000).

Weak anion exchange (WAX) columns are packed with a porous resin containing diethylaminoethyl (DEAE). The strong anion exchange (SAX) columns on the other hand are packed with resins containing quaternary alkylamines. The different properties of these column types are especially relevant for the resolution of phosphorothioate (PS) ODN analogues (non-bridging oxygen atoms replaced by sulphur atoms). The SAX column is generally employed if the PS ODN contains 10 nucleotides or less while longer ODNs are resolved with WAX columns. The strong association between the longer PS-ODNs (>10 nucleotides) and the SAX resin necessitates the use of strong halide displacers (LiBr or LiCl) in the mobile phase instead of the ordinary phosphate, sulphate or perchlorate containing mobile phases (Metlev and Agrawal, 1992; Borque and Cohen, 1993).

3.1.2.2. Reversed phase HPLC (RP HPLC)

In RP HPLC the elution order of the molecules to be separated is determined by their hydrophobicity. Usually the hydrophobicity of the mobile phases used to elute the sample from the column, is controlled by the addition of water miscible organic solvents such as alcohols or acetonitrile.

Phosphorothioate (PS) ODNs longer than 10 bases are not easily purified on reversed phase columns due to their increased hydrophobicity compared to phosphorodiester (PO) ODNs (Agrawal *et. al*., 1990; Thayer *et. al*., 1996). As a result, PS ODNs are usually separated by anion exchange HPLC (Agrawal *et. al*., 1990). Reversed phase HPLC has been shown to be very useful for the purification and analysis of PO ODNs provided that the negative charge of the phosphate groups in the phosphodiester backbone is first neutralized by a counter-ion such as triethylammonium (ion-pair reversed phase chromatography; Kwiatkowski *et al.*, 1984).

The latter method was utilized to determine the stability of the modified PO ODN, ASL-1, in culture medium. It was assumed that all the ODNs would exhibit similar properties to ASL-1 due to the equivalent length, similar base composition and the same 3'-self-complementary loop modification. The separation and stability of only ASL-1 (designed in chapter 2) is therefore described below.

3.2. Materials and Methods

3.2.1. **Materials**

Reversed phase C_{18} columns were purchased from Phenomenex (Torrance, California, USA). Triethylammonium acetate (TEAA) was purchased from Fluka as a 1 M stock solution (pH 7.0). Acetonitrile, chloroform, diethylether, ethanol, isoamyl alcohol, methanol (all analytical grade), D-glucose, EDTA, hypoxanthine, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydrogen carbonate and TRIS were all purchased from Merck. Glycogen, proteinase K, LiCl and oligonucleotides were obtained from Roche Diagnostics. *Sec*-butanol, RPMI-1640, L-glutamine, gentamycin and HEPES buffer were purchased from Sigma.

3.2.2. **Methods**

3.2.2.1. Optimization of separation and elution conditions of ODN ASL-1 on reversed phase HPLC

The C_{18} column was conditioned and equilibrated with 0.1M triethylammonium acetate (TEAA) in 80% acetonitrile according to the manufacturer's instructions. TEAA is used as counter-ion in ion pair chromatography and was applied in these studies.

3.2.2.2. HPLC analysis of ODN samples

A Varian 9012 HPLC system (Waters) was used in these investigations. A Phenomenex LUNA C_{18} column (dimensions: 150 x 4.60 mm) was employed. Elution buffer A consisted of 0.1M TEAA and buffer B of 0.1M TEAA supplemented with varying

percentages of acetonitrile. Various gradients were investigated to optimize the elution conditions (Table 3.1).

For these investigations ODNs were injected onto the C_{18} column directly. Elution was conducted at a flow speed of 1 ml/min and monitored at a wavelength of either 260 nm or 270 nm (Borque and Cohen, 1983; Deshmukh *et. al*., 2000).

3.2.2.3. Method used for extraction of ODNs from parasite culture medium

3.2.2.3.1. Fluid phase extraction

For this extraction 0.25 µg ODN (dissolved in 0.5 µl double distilled, sterile water) was incubated at 37 °C for time periods specified for each experiment in 250 µl serum-containing culture medium. For extraction 250 µl DNA extraction buffer (50 mM SDS, 10 mM NaCl, 20 mM Tris, 10 mM EDTA, pH 9.0 and 20 µl of 10mg/ml proteinase K dissolved in 10 mM Tris (pH 7.5) for the digestion of serum proteins (Borque and Cohen, 1993) was added. Following incubation at 60°C for 90 minutes, 600µl chloroform/phenol/isoamyl alcohol (25:24:1) was added next, followed by vigorous mixing for 15 seconds. After centrifugation at 14 000g for 2 minutes the upper aqueous phase was removed and successively washed twice with 1 ml *sec*-butanol and once with 500 µl diethylether interspaced with a vortexing and centrifugation step as above. After removal of the upper phase, the ODN containing aqueous phase was allowed to air dry and the residue was suspended in 1 ml of 0.1 M TEAA buffer immediately prior to HPLC (Borque and Cohen, 1989).

An internal standard (IS), containing the first 15 nucleotides of the ASL-1 ODN designed in chapter 2, was included in all extractions but not incubated in culture medium. To determine the stability of ASL-1 in culture medium, the peak height of the ASL-1 (sample) ODN, incubated in culture medium for various periods of time, was expressed as a ratio of the peak height of the IS ODN, added after incubation. A decrease in this ratio would be indicative of ODN breakdown after a specified incubation time. The RP HPLC gradient conditions used in these investigations are given below in Table 3.1.

Table 3.1: The gradient conditions used to establish successful separation between ASL-1 and internal standard (IS). Buffer B: 0.1 M TEAA, 80% acetonitrile.

Mobile Phase and gradient composition	**Time period for each step (min)**	**Time at onset of each step (min)**
0% Buffer B	5.00	0.00
0% - 50% Buffer B	90.00	5.00
50% Buffer B	2.00	95.00
0% Buffer B	1.00	97.00
END		98.00
Data collection stopped after 90 minutes		

Depending on the separation distances of IS and ASL-1 it is possible to observe the appearance of degradation products only between the respective lengths of these two ODNs. This in turn would enable comparison of the results obtained with the half lives of unmodified PO ODNs as reported in the literature. ODN recoveries given in the results

were calculated by substituting the peak heights, obtained after each appropriate HPLC analysis into the equation for the standard curve (y = 85829.78x + 907.1053) and solving for the ODN concentration. This resulted in the following equation to be solved: x = (y-907.1053)/85829.78. This was done for both ASL-1 and SL-1.

3.3. Results

3.3.1. Extraction of ASL-1 and IS from serum containing medium and subsequent elution conditions from the C_{18} reversed phase HPLC column

The fluid-phase extraction method was used since standard extraction methods incorporating ethanol precipitation steps were not successful (Borque and Cohen, 1993). The method was used successfully by the latter authors for the extraction of 25-mer PS ODNs from human serum and urine. In the experiments reported here the phosphodiester ODNs were extracted from parasite culture medium and quantitated by ion pair reversed phase HPLC. ODNs were extracted after incubation at 37^{o}C for time periods ranging from 0-6 hours.

The detector wavelength was set to 270 nm so as to prevent possible interference from traces of *sec*-butanol used during the extraction process (Borque and Cohen, 1993, Niederwieser and Brenner, 1965). Several gradient conditions were investigated and the gradient settings that attained the most satisfactory separation are given in Table 3.1 (Methods). The separation achieved under these conditions is shown in Figure 3.1.

The separation time between the two ODNs is 2.29 min, with the IS eluting at 36.30 minutes and ASL-1 at 38.59 minutes. No peaks were observed after extraction of incubation medium without prior addition of ODN (results not shown).

To determine the extent of ODN degradation under incubation conditions, the above extraction and HPLC conditions were applied in the next series of experiments with ASL-1.

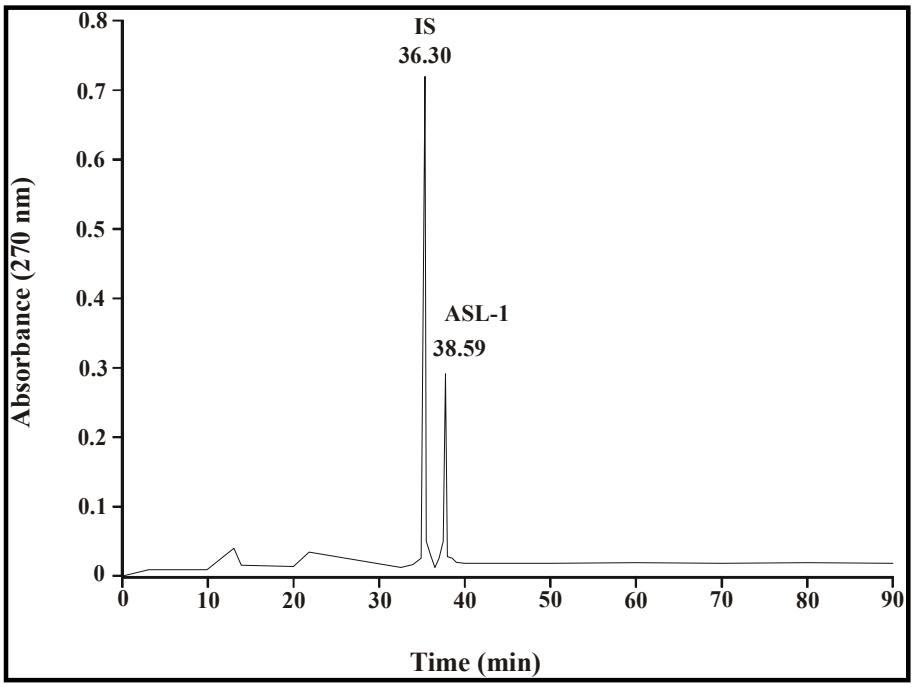

Figure 3.1: A typical reversed phase HPLC chromatogram (Varian 9012 HPLC) obtained using a Phenomenex LUNA C_{18} column. The antisense (0.3 µM) ODN ASL-1 was incubated at 37 °C for four hours followed by extraction of ASL-1 and IS (0.9 µM) ODNs using the fluid phase extraction procedure. Gradient conditions used are given in Table 3.1.

3.3.2. Determination of ODN stability

A calibration curve of peak height versus ASL-1 concentration was set up and is shown in Figure 3.2.

The calibration curve had a correlation coefficient (r^2) of 0.9985, which was indicative of linear ODN detection. The ODN, ASL-1 was incubated at 37°C in serum-containing culture medium from one to six hours, extracted along with the internal standard added at the end of the incubation period, and separated by reversed phased C_{18} column chromatography. The results are shown as the ratio of sample to standard peak height, plotted against incubation time (Figure 3.3).

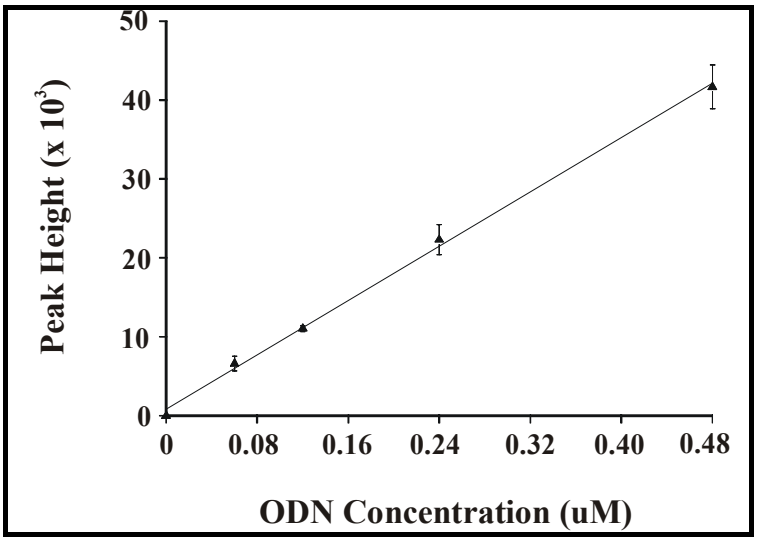

Figure 3.2: Calibration curve (r^2 = 0.9985) showing the relationship between sample ODN peak height obtained after HPLC and ODN concentrations ranging from zero to 0.48 µM. Each data point is expressed as the average ± SEM of three repeats. Antisense ODN ASL-1 was directly injected at the given concentrations. The straight line equation was y = 85829.78x + 907.1053.

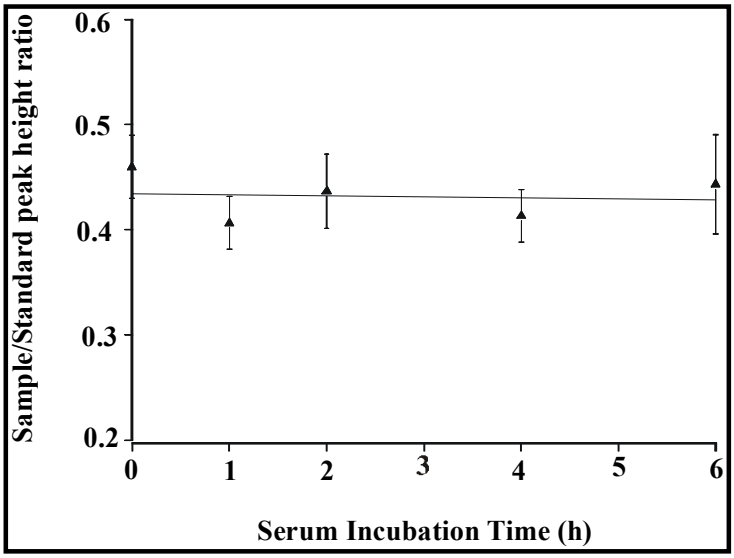

Figure 3.3: Stability of a modified phosphodiester antisense ODN, targeted against *P. falciparum* α-I-tubulin, after incubation in serum-containing medium for time periods ranging from zero to six hours. Prior to extraction and subsequent HPLC analysis, internal standard ODN (0.9 µM) was added to the incubated sample ODN (ASL-1) (0.3 µM). The sample/standard ODN peak height ratio was determined with increasing incubation time to assess ASL-1 stability. Each data point is expressed as the mean ± SEM of three repeats.

The average ratio of sample peak height to internal standard peak height over the 6 hour incubation period was 0.43 ± 0.024. This result is similar to a ratio of 0.44 ± 0.078 obtained for non-incubated, non-extracted ODNs (results not shown), suggesting that the modified antisense phosphodiester ODN, ASL-1 is stable up to 6 hours after incubation in serum containing medium.

The recoveries of both ASL-1 and IS after HPLC analysis were determined after each incubation and are shown in Table 3.2.

Table 3.2: The recoveries of both ASL-1 and IS ODNs from culture medium after the specified incubation periods of ASL-1, followed by fluid phase extraction of both ASL-1 and IS.

Incubation time of ASL-1(h)	**ASL-1 recovery (%) ± S.E.M**	**IS recovery (%) ± S.E.M**
1	12 ± 1.41	12 ± 1.89
2	11.2 ± 1.84	9.9 ± 1.50
4	9.7 ± 3.60	9.6 ± 4.20
6	11 ± 0.80	9.9 ± 0.78

From the table above it is noteworthy that the recoveries of both ASL-1 and IS after fluid phase extraction and HPLC analysis, differ from each other by no more than 1,3% for each specific incubation time.

3.4. Discussion

Nuclease degradation of unmodified phosphodiester ODNs is a problem in antisense studies since both sequence specificity and efficacy are compromised (Crooke, 2000). This is due to the fact that shorter ODN segments are more likely to be complementary to parasite mRNA sequences other than the target sequence as well as to host sequences. In order to prevent or limit degradation of ODNs several modifications are employed as were described in Chapter 1. The structurally modified ODN, ASL-1 targeted the upstream and downstream regions including the translation initiation codon of *P. falciparum* α-I-tubulin. The other two ODNs targeted downstream regions of α-I-tubulin mRNA and were designed using computer-based predictions of the most favourable hybridisation sites. All three ODNs were of the same length and included a 3'-hairpin loop for protection against 3' exonuclease degradation. After ASL-1 incubation in culture medium it was necessary to extract the ODN prior to HPLC analysis in order to remove the unwanted protein and lipid components of the medium.

A fluid-based extraction method was used since the standard precipitation methods using ethanol are designed to eliminate ODNs having the length of ASL-1 (Sambrook *et. al*., 1983). The method followed made use of a protein digestion step prior to a standard extraction with phenol/chloroform/isoamyl alcohol. This was followed by two successive *sec*-butanol washes, the purpose being to remove traces of phenol, lipid-soluble components as well as water. An extraction with diethylether was included to remove most of the *sec*-butanol and phenol from the concentrated ODN extract. This method was

considered applicable since it was first used to extract PS modified ODNs from human urine and serum (Borque and Cohen, 1993). Figure 3.2 showed that extracted ASL-1 was detected when using the HPLC method described.

The HPLC method was chosen in preference to capillary electrophoresis (CE) due to the additional purification steps required for samples extracted from biological media, prior to CE analysis (Bansal *et. al*., 2001; Leeds *et. al*., 1996). Reversed phase rather than anion exchange HPLC was the preferred method since the former was shown to be more suited for the purification and analysis of phosphodiester ODNs (Agrawal *et. al*., 1990; Bansal *et. al*., 2001; Bourque and Cohen, 1993; Toulme *et. al*., 1996). Elution of ASL-1 from the C_{18} column was achieved using 0.1 M TEAA, a standard ion-pairing reagent as mobile phase A and mobile phase B being supplemented with 80 % acetonitrile (Figure 3.1). These were standard elution conditions for PO-ODNs from this type of column (Apffel *et al*., 1997; Toulme *et al*., 1997). However, sample to sample variation made the use of an internal standard imperative. It was decided to use a 15-mer ODN (IS) for this purpose and to adjust the HPLC gradient such that ASL-1 and IS were completely separated (Figure 3.1).

The sample (ASL-1)/standard (IS) peak height ratios, obtained after the various ASL-1 incubation times, were calculated and plotted against ASL-1 incubation time. A decrease in this ratio over the 6 hour incubation period would be indicative of ASL-1 degradation. In this regard it is important to note that it is advantageous to make use of peak height rather than peak area. ODN degradation could give rise to a decrease in the peak height

accompanied by a simultaneous peak broadening. In this instance no change in the peak area would be measured and quantification of the intact would be problematic.

This chosen method is therefore capable of indicating the stability of a modified ODN over an unmodified counterpart. Different methods would have to be employed to accurately determine the degradation of a modified ODN under specified conditions over time. These methods would include capillary electrophoresis, which allows for the separation and detection of ODNs differing in length by only a single nucleotide.

After ASL-1 incubation in culture medium for 1, 2, 4 and 6 hours, respectively, ASL-1/IS peak height ratios were plotted against time of ASL-1 culture medium incubation (Figure 3.5). The results indicated that ASL-1 was not degraded after 6 hours of incubation in culture medium. This result is further supported by the similar recovery values calculated for both ASL-1 and IS over the 6 hour ASL-1 incubation period (Table 3.3). The observed stability of the modified PO-ODN is significant since the half-life of an unmodified PO-ODN in culture medium is in the range of minutes (Gilar *et. al*., 1997; Phillips and Zhang, 2000).

The observed recovery of ODNs from culture medium is consistently low and thereby highlight the difficulties associated with retrieving short ODNs from a variety of biological media (Borque and Cohen, 1993; Sambrook *et. al*., 1983). The extraction procedure performed in these experiments allowed for HPLC analysis of ODNs (ASL-1 and IS) after incubation in culture medium to determine the stability of the structurally

modified ASL-1. It must however be noted that the studies performed give no indication of ASL-1 half-life in culture medium or possible cleavage of smaller numbers of nucleotides (2-3 nucleotides for example) from the full length ODN. To obtain this information would require improvements in the efficiency of the extraction method and a method such as capillary electrophoresis to separate starting material from degraded products. A detailed analysis of each step of the extraction process could be undertaken in order to determine where most of the ODN is being lost. If the problem should prove to be in the actual extraction process it may be necessary to investigate the effect of changes in the composition of the extraction buffer (SDS, NaCl and proteinase-K) on the extraction efficacy of the method. It is also possible that extracted ODNs are being lost during the wash steps with *sec*-butanol and/or diethylether.

The inclusion of an unmodified PO-ODN, having the same sequence as ASL-1 but lacking the structural modification, would furthermore enable a direct comparison between the stabilities of the respective ODNs in culture medium as well as differences in inhibitory efficacy between the two ODNs.

The results obtained have however, shown that further studies on ASL-1, including inhibitory efficacy, are warranted. The results of investigations on the uptake of the modified antisense ODNs in relation to their inhibition of parasite propagation in erythrocyte cultures are presented in the next chapter.