The Determination of Therapeutic Oligonucleotides in Blood Plasma by Capillary Gel Electrophoresis with Laser-Induced Fluorescence Detection

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Abstract

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Capillary gel electrophoresis (CGE) is the premier method of separating DNA and oligonucleotides. This technique is the workhorse method of all separations based genomics studies e.g. Human Genome Project. In previously published studies, methods utilizing CGE with ultraviolet (UV) detection have been developed for the quantitative determination of oligonucleotides in various matrices (e.g. plasma, serum, tissue); although very selective CGE-UV is not as sensitive as other analytical techniques (e.g. hybridization assays). Recently, laser-induced fluorescence (LIF) detection has emerged as a significantly more sensitive alternative to UV detection. However, oligonucleotides do not possess significant native fluorescence, therefore covalent or non-covalent dying is necessary. OliGreen™, an ultra sensitive fluorescent nucleic acid stain, non-covalently binds to single stranded-oligonucleotides (ss-oligo's) and displays a significant fluorescence enhancement and is potentially the most sensitive dye for ss-oligos. The purpose of this study was to develop and validate a method for the quantitative determination of therapeutic ss-oligos in plasma. Plasma was spiked with ss-oligos (18, 22, 23 and 24-mer) and separation was achieved by CGE. The oligonucleotides were non-covalently complexed by mixing OliGreen™ (1/500 dilution) into the gel separation matrix at a temperature of 60°C, the maximum of the instrument. Detection limits of 250 pg/mL and linear calibrations over 100 orders of magnitude were achieved using the 488

nm argon ion laser for excitation and 520 nm for detection. The method was validated with respect to selectivity, carry-over, linearity, intra- and inter-assay precision and accuracy.

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I would like to dedicate this thesis to my grandmothers, Farida Khadjadourian and Azniv Aghbabian, as well as Sami Hantash who parted this world too soon and did not get a chance to seeing this day. You will never be forgotten.

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Chapter 1

Introduction

1.1 Purpose/Scope of Work

Cancer, Human Immunodeficiency Virus (HIV), Alzheimer's, Parkinson's; all serious diseases which have unfortunately become words we hear in our everyday's lives. Many of these diseases are associated with the over-expression or the abnormal expression of a gene that results in overproduction or abnormal production of a protein. Proteins are fundamental components of all living organisms and play a crucial role in virtually all biological processes. Many types of proteins exist (enzymes, hormones, antibodies), all of which are necessary for carrying out a cell's basic functions, such as enzymatic catalysis, growth control, cell differentiation, protein transport and storage.[1] The majority of drugs on the market today (small molecules and biologics) bind to proteins and alter their biological activities thus inhibiting certain diseases. Recently, DNA-based biopharmaceuticals have emerged as a new class of therapeutic drugs which target disease progression at the genomic level (gene therapy). The basic idea behind gene therapy is to target nucleic acids (DNA and RNA) at different levels of gene expression using compounds of similar nature so that disease associated proteins can be prevented from forming.[2-4] Synthetic oligonucleotides are one category of this new class of therapeutic agents. These oligonucleotides have a wide range of modes of action, for example antisense oligonucleotides selectively hybridize to their target complementary mRNA via Watson-Crick base pairing.[2-4] Different types of therapeutic

oligonuceolotides exist and include plasmids containing transgenes, single stranded antisense oligonucleotides (ss-ASO), ribozymes, DNAzymes, aptamers and small interfering RNAs (siRNAs).[5-7] DNA based therapeutics are very potent drugs which is why the dose levels introduced to humans is low.

The purpose of the work presented here was to develop and validate a method for the determination of therapeutic oligonucleotides, more specifically single-stranded antisense oligonucleotides, in blood plasma using capillary gel electrophoresis coupled using laser induced fluorescence detection. In previously published studies, methods utilizing CGE with ultraviolet (UV) detection have been reported for the quantitative determination of oligonucleotides in various matrices (e.g. plasma, serum, tissues).[8] Although very selective (separation of intact oligonucleotides as well as related metabolites that differ by only one or two base units), CGE-UV is not as sensitive as other analytical techniques (e.g. hybridization assays). Recently, LIF detection has emerged as a significantly more sensitive alternative to UV detection for CGE applications.

1.2 Introduction to DNA-based Therapeutic Drugs

1.2.1 Historical Review of Deoxyribonucleic Acid

In 1953, James Watson and Francis Crick (Figure 1-1) published an article in the journal Nature on the double-helical structure of deoxyribonucleic acid (DNA) and were later awarded the Nobel Prize in 1962 for their revolutionary discovery. [9-10] Their discovery was the culmination of a decade of intense research following Avery, MacLeod and McCarty's demonstration that DNA is the fundamental molecular storage center of all

heritable genetic information.[11] Today, DNA has become the icon of the modern biosciences.

Cambridge duo: Francis Crick (left) and James Watson at the Cavendish Laboratory, Cambridge. The publication of their proposed structure for DNA was published 50 years ago this month. (Picture: Science Photo Library.)

Figure 1-1: Picture of Francis Crick (left) and James Watson[12]

Understanding its structure and how it functions has fundamentally changed our world, for example, genetic testing, forensics and genetically modified organisms. Most of modern biology relies heavily on molecular genetic techniques. What seems as a complex term to the average individual, DNA, to the scientific community, is simply an organic polymer. The DNA molecule is made of a long string of chemical building blocks called nucleotides. There are four different types of nucleotides found in DNA, adenine (A), guanine (G), cytosine (C) and thymine (T). Ribonucleic Acid (RNA) has a similar structure to DNA, with the exception of one carbon from the sugar moiety which carries an OH group and thymine replaced by uracile (U). Each nucleotide is made of three components: a phosphate backbone, deoxyribose sugar moiety and a nitrogenous

base as shown in Figure 1-2 and Figure **1-3**. The phosphate backbone and sugar moiety remain unchanged between nucleotides while the nitrogenous base changes as to distinguish between each nucleotide.1 Nucleic acids have the innate property to hybridize with each other through hydrogen-bonding (H-bonding), via Watson-Crick base-pairing. Adenine will pair with thymine (or uracile in RNA) via two H-bonds, whereas guanine will pair with cytosine via three H-bonds (Figure 1-3 and Figure **1-4**).

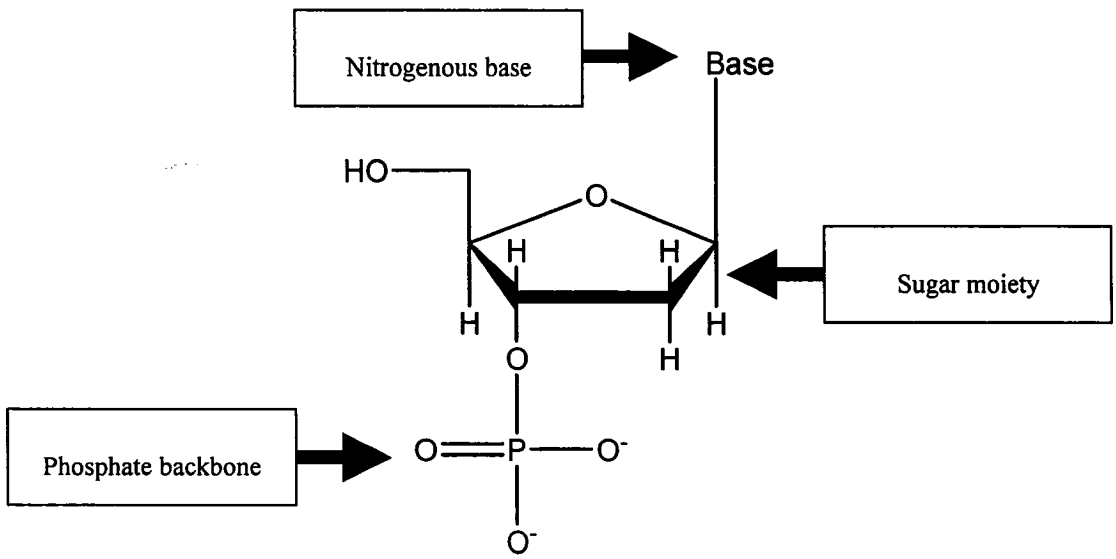


Figure 1-2: Deoxyribonucleic Acid Structure (DNA). Each nucleotide made of three components: a phosphate backbone, deoxyribose sugar moiety and a nitrogenous base.

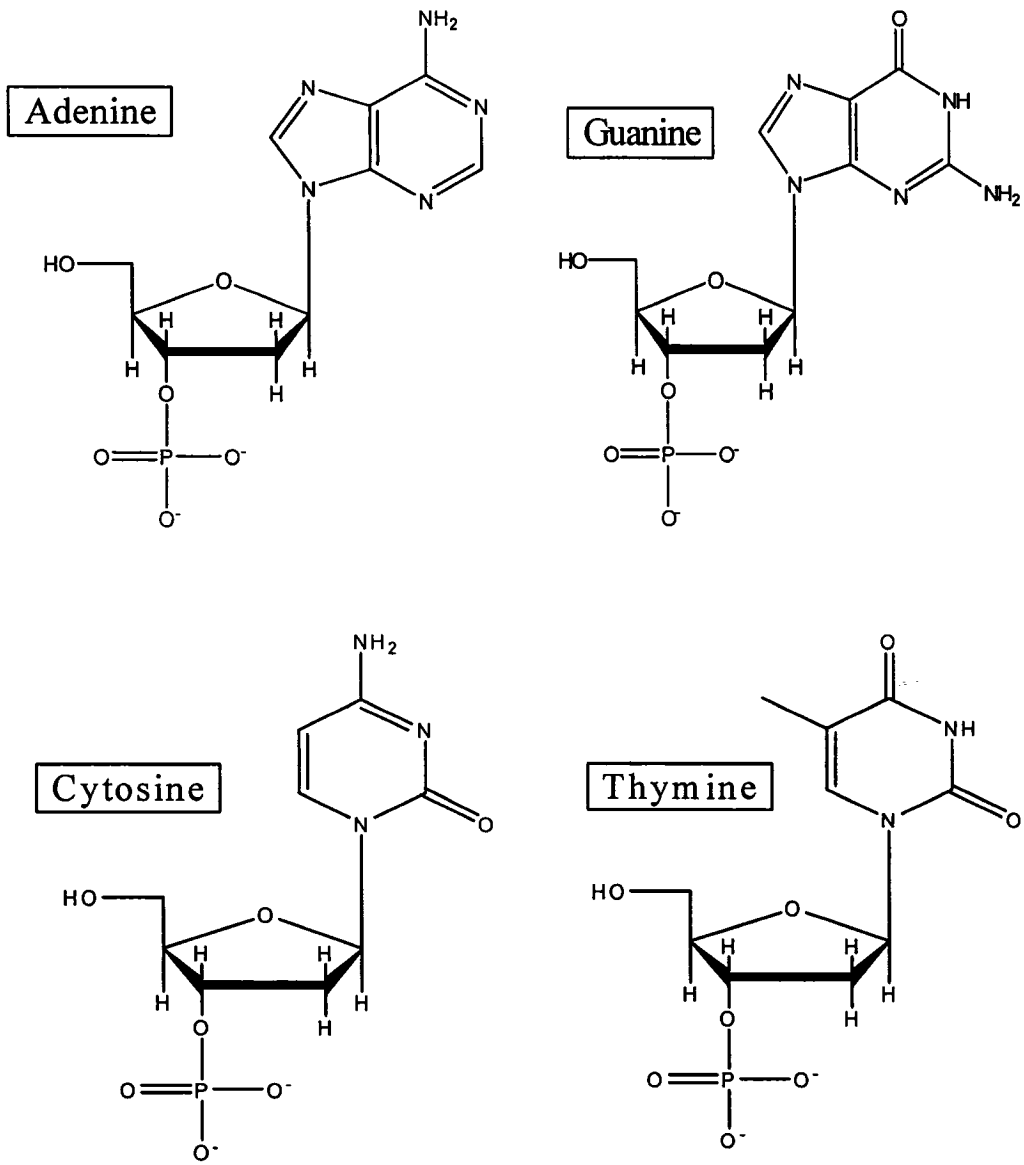


Figure 1-3: Nucleotide Structure: adenine (A), guanine (G), cytosine (C) and thymine (T).

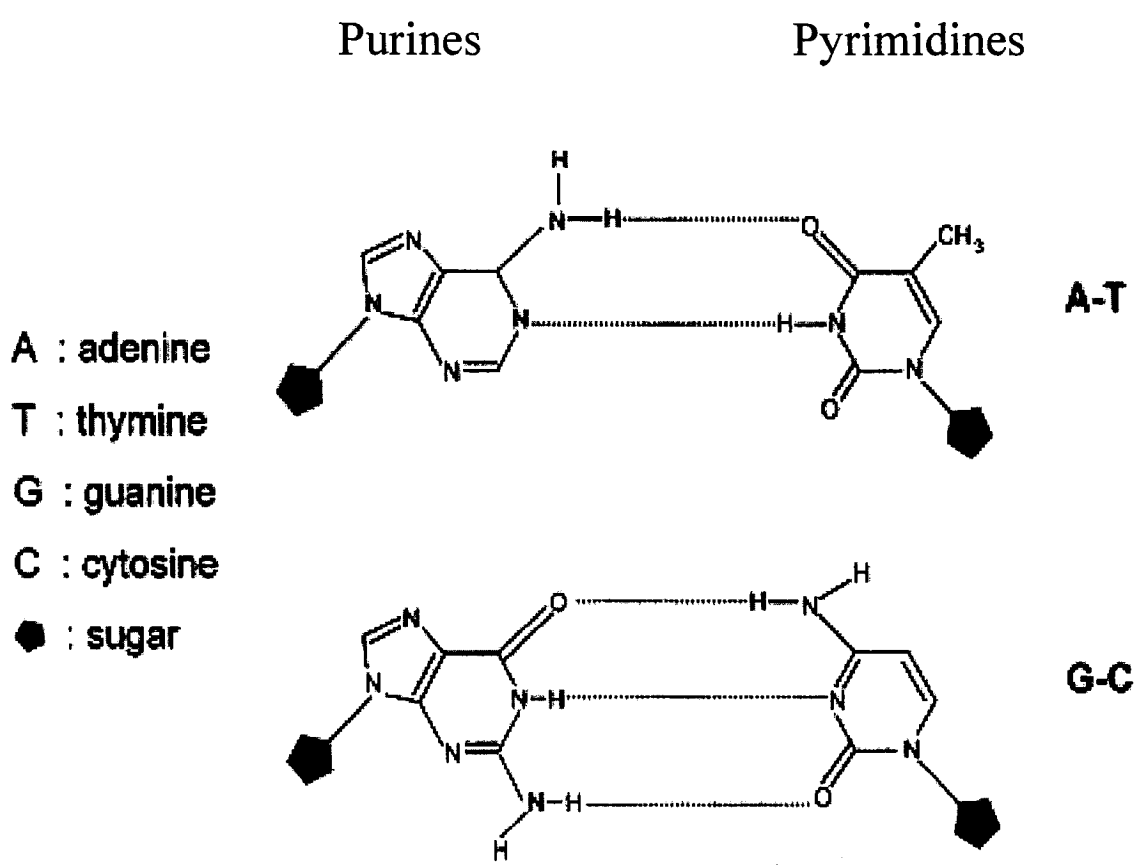


Figure 1-4: Watson-Crick hybridization. Nucleic acids hybridize with each other through hydrogen-bonding, via Watson-Crick base-pairing. Adenine will pair with thymine (or uracile in RNA) and guanine will pair with cytosine via three H-bonds.[13]

1.2.2 Single-Stranded Antisense Oligonucleotides

In 1978, Zamecnik and Stephenson were the first to discover the potential of a synthetic oligodeoxynucleotide to inhibit the Rous sarcoma viral replication and cell transformation in chick embryo fibroblast tissue cultures.[14] In 1996, Zamecnik was awarded the Lasker prize for his work and contribution to the scientific community. Since their discovery, antisense technology has developed into a promising therapeutic tool in modern medicine.

Antisense oligonucleotides (ASO) are given the "antisense" term because they are the opposite "sense" of the original RNA or DNA strand. They are short stretches of DNA, usually 12 to 28 nuclotides that hybridize to target mRNAs (corresponding to very specific disease-causing genes) via Watson-Crick base pairing.[2-4,15] By binding to the mRNA, antisense oligonucleotides block the production of the genes into disease-causing proteins (Figure 1-5).

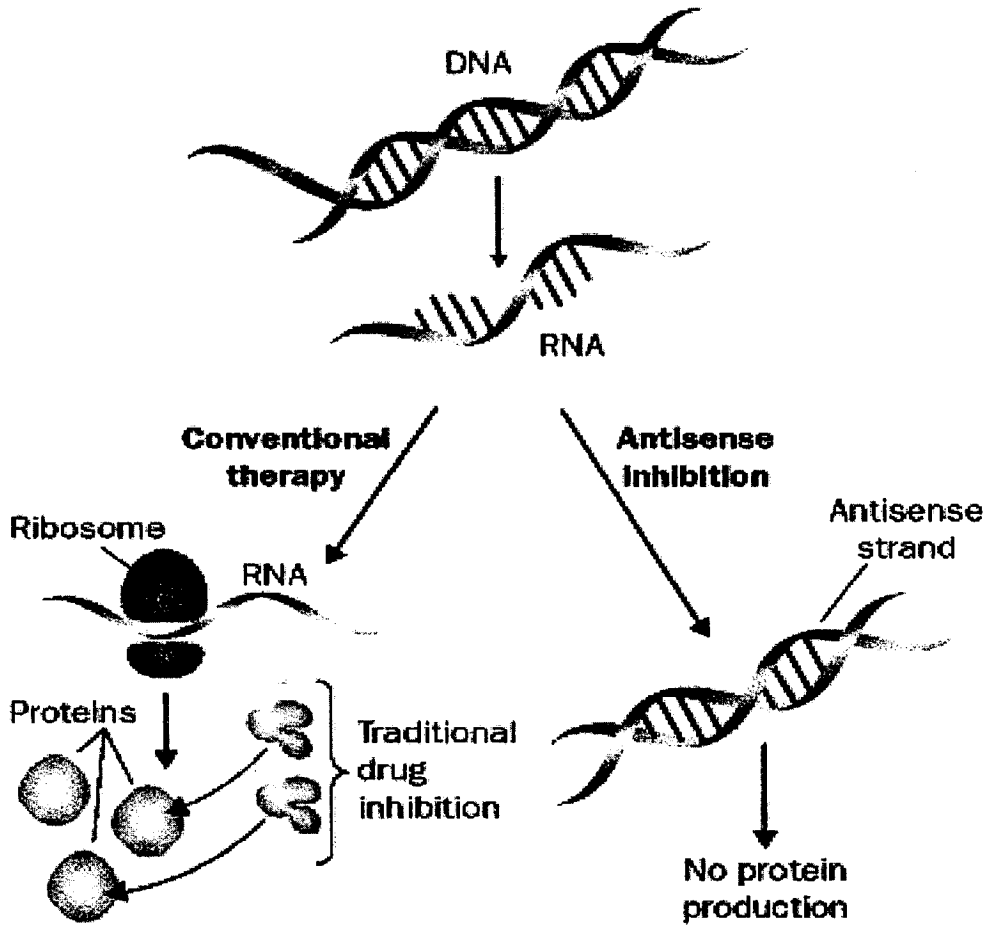


Figure 1-5: Antisense oligonucleotides: basic mechanism of action. Traditional drugs bind to proteins to inhibit diseases whereas ASOs target disease progression at different levels of gene expression using compounds of similar nature so that disease associated proteins can be prevented from forming.[16]

Antisense therapy is thus an attractive alternative to classic competitive antagonist drugs since it can potentially provide highly specific results, with few toxic effects, for safe and effective therapeutics of a large number of diseases including various types of cancers, AIDS, diabetes, Crohn's disease, psoriasis, multiple sclerosis, muscular dystrophy, asthma, rheumatoid arthritis, hepatitis and chronic cardiovascular diseases. The high specificity and selectivity of this new class of therapeutic drugs is based on the fact that

any sequence within the human genome which is composed of a minimum of 17 consecutive DNA bases (or a minimum of 13 consecutive bases for RNA) is repeated only once.[17-18]

1.2.2.1 Cellular Uptake

For this new generation of synthetic therapeutic drugs to be efficient, they must first enter the cell nucleus. There is a general lack of basic understanding regarding the cellular uptake of these oligomers.[19] However, the proposed and widely accepted mechanism of oligonucleotide internalization into the cytoplasm is via endocytosis, inward folding of the plasma membrane or invagination, although this can vary depending on the oligonucleotide structure.[20-22] Specific endocytosis mechanisms have also been identified as potential routes of olignucleotide internalization such as pinocytosis, receptor-mediated endocytosis and adsorptive endocytosis.[17] Delivery of ASO to their corresponding targets remains a challenge since once inside the cell, the oligonucelotides are likely to be encapsulated by endosomes and still susceptible to lysosomal degradation (lysosomes contain many hydrolytic enzymes). To circumvent the barriers of cellular uptake and at the same time increase the stability of the oligonucleotide within the cell, researchers have focused their efforts on developing delivery systems that include, but are not limited to, encapsulation within liposomes or cyclodextrins, complexation of oligodeoxynucleotides (ODNs) with cationic compounds or use of nanoparticles as carriers for these molecules, etc. [19, 23-25]

1.2.2.2 Mechanism of action

Multiple mechanisms exist by which synthetic oligonucleotides inhibit gene expression in human cells, however two major mechanisms are reported. The most widely exploited mechanism of action of ASOs is by activation of RNase-H. RNase-H is a ubiquitous ribonuclease that cleaves the 3'-O-P-bond of RNA in a DNA/RNA duplex to produce 3'-hydroxyl and 5'-phosphate terminated product and therefore leads to the degradation of the target mRNA.[1] This non-specific endonuclease catalyzes the cleavage of RNA via a hydrolytic mechanism.[26-27] Once the ASO hybridizes to their RNA target, the heteroduplex formed by the oligonucleotides and its target can be recognized by RNase-H and thereby be degraded.[28]

The second mechanism of action of ASOs is by translational arrest. By hybridizing to the complementary strand of mRNA, the ASO physically blocks the site thus preventing access and binding of various factors such as ribosomes. Blocking the ribosome, the protein-translational machinery, at any one of the three steps required for translation can potentially interrupt protein synthesis.[28]

1.2.2.3 Chemical Modifications to Backbone

As discussed in the previous paragraphs, unmodified oligodeoxynucleotides are unstable and rapidly degraded in biological fluids by nucleases. A variety of modifications have been proposed in order to stabilize these molecules such as phosphate backbone modifications, sugar moiety modifications as well as using unnatural bases as analogs of natural bases.[15] Based on the available literature, chemical modifications have mainly focused on phosphate backbone modifications and sugar moiety modifications. Based on these modifications, three generations of antisense oligonucleotides can be distinguished.

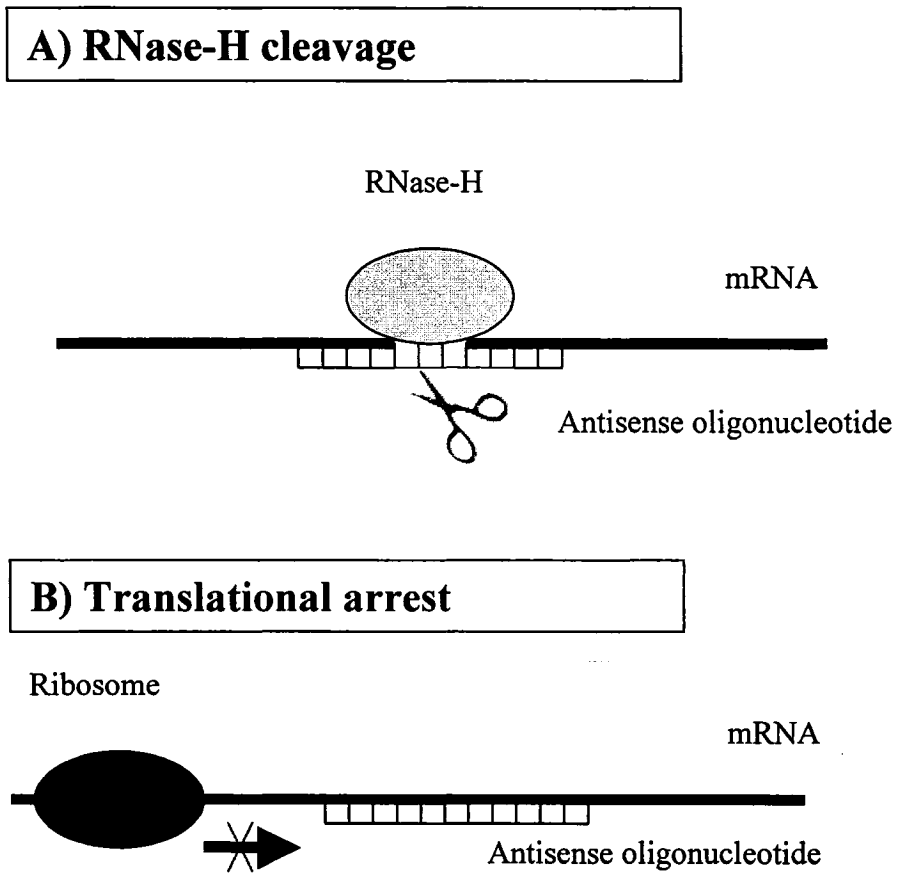


Figure 1-6: Mechanism of antisense activity: A) RNase-H cleavage induced by ASOs. B) Translational arrest by blocking the ribosome.

First Generation Antisense Oligonucleotides

Phosphothioates are probably the earliest and best known DNA analogs to date.[15,17] In phosphothioate oligonucleotides, one of the non-bridging oxygen atoms is replaced by a sulphur atom (Figure 1-7).

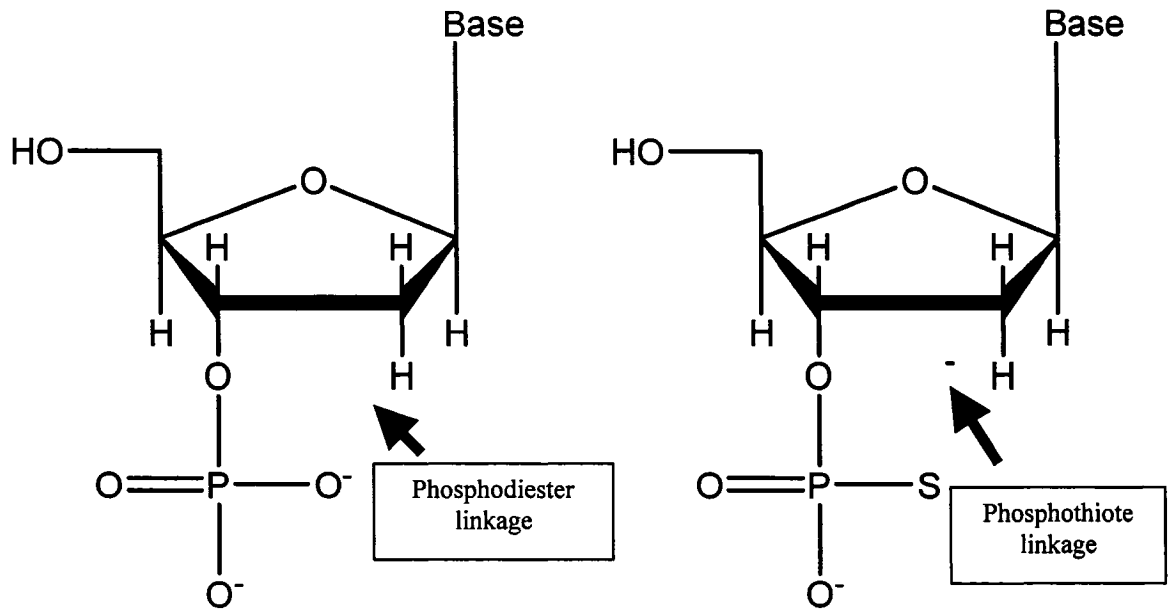


Figure 1-7: Depicted is the structure of a first generation antisense oligonucleotide: a phosphotioate oligonucleotide. One of the non-bridging oxygen atoms is replaced by a sulphur atom.

The substitution of the sulphur atom enhances the nuclease stability of the ASO.[29] Studies have shown that phosphothioate oligonucleotides have a half-life of approximately 9-10 hours in human serum in comparison to phosphodiesters that have a half-life of approximately 1 hour.[30] The mechanism of action of phosphothioates is by activation of RNase-H.[2-3] Phosphothioates also carry a negative charge that is unsymmetrically distributed and located mainly on the sulfur atom that plays an important role in cellular delivery.[15-17] Phosphothioates tend to bind non-specifically to certain plasma proteins, which is a major disadvantage since it can lead to cellular toxicity.[31]

Second and third Generation Antisense Oligonucleotides

To circumvent, to some degree, the issues that affected first generation antisense oligonucleotides, researchers came forth with newer modifications. Some examples of these molecules are 2'-*O*-methyl and 2'-*O*-methoxyethyl RNA, peptide nucleic acids (PNAs), locked nucleic acids (LNAs), N3'-P5' phosphoroamidates (NPs), 2'-deoxy-2'-fluoro-beta-D-arabino nucleic acids (FANA). For an extensive and in-depth review of DNA-based biopharmaceuticals including antisense oligonucleotides, the reader should refer to the articles written by Kurreck[15], Aboul-Fadl *et al.*[32]and Crooke.[2-3]

1.3 Analytical methods for Quantifying Antisense Oligonucleotides

Vitravene, a phosphothioate antisense oligonucelotide developed by ISIS Pharmaceuticals (Carlsbad, CA) designed to inhibit the replication of cytomegalovirus retinitis in HIV patients, was the first of this class of compounds to be approved by the Food and Drug Administration (FDA).[33-34] Since the commercialization of Vitravene, many biotechnology companies, including Genta, Epigenesis, Lorus, AVI Biopharma, Hybridon, *etc.*, started developing antisense drugs. Many of these drugs are currently in the pre-clinic as well as various phases of clinical trials.

Table 1-1: Antisense oligonucleotides in various phases of clinical trials

Drug	Company	Target / Disease	Chemistry	Development phase
ISIS-3521 (Affinitac)	ISIS Pharmaceuticals	PKC-α / cancer	Phosphotioate	Phase III
ISIS-5132	ISIS Pharmaceuticals	c-RAF / solid tumour	Phosphotioate	Phase I/II
ISIS-2503	ISIS Pharmaceuticals	H-ras / solid tumour	Phosphotioate	Phase I/II
ISIS-2302 (Alicaforsen)	ISIS Pharmaceuticals	ICAM-1 / Psoriasis, Crohn's disease, Ulcerative colitis	Phosphotioate	Phase II/III
ISIS-14803	ISIS Pharmaceuticals	Antiviral / Hepatitis C	Phosphotioate	Phase II
OGX-011	OncoGenex	Clusterin / prostate cancer	2'-O-(2-methoxy)ethyl -gapmer	Phase I/II
GTI-2040	Lorus Therapeutics	Ribonucleotide reductase (R2) / cancer	Phosphotioate	Phase II
GTI-2051	Lorus Therapeutics	Ribonucleotide reductase (R1) / cancer	Phosphotioate	Phase I
EPI-2010	Epigenesis	Adenosine A1 receptor / asthma	Phosphotioate	Phase II
Avi4126	Avi BioPharma	c-myc / cancer, kidney disease	3rd generation	Phase I/II
Avi4557	Avi BioPharma	CYP3A4 / metabolic redirection of approved drugs	3rd generation	Phase I
Gem231	Hybridon	PKA RIα / solid tumors	2nd generation	Phase I/II
Gem92	Hybridon	HIV gag / AIDS	2nd generation	Phase I

The development of ASOs into therapeutic agents has prompted the development of bioanalytical methods for their quantitation in various biological fluids and tissues. These methods are necessary for the characterization of the pharmacokinetic (PK) and pharmacokinetic/pharmacodynamic (PK/PD) behaviour of these drugs in support of pre-clinical and clinical development. The most important bioanalytical methods currently available for the quantitation of antisense oligonucleotides in various biological matrices include radiotracer methods, high-performance liquid chromatography (HPLC), traditional gel electrophoresis, capillary gel electrophoresis with UV or LIF detection, HPLC-mass spectrometry, and hybridization-based enzyme-linked immunosorbent assays.[35] These various techniques can be ranked based on their reproducibility, selectivity, sensitivity, precision and accuracy and their cost per sample. For a method to be suitable for the accurate PK and PK/PD characterization of ASOs, it should be able not only to quantify the parent oligonucleotide, but it's chain-shortened metabolites (N-1, N-2, *etc.*) as well, with great sensitivity.

Radiolabel tracer methods

Labelling oligonucleotides with radiotracers followed by scintillation counting for detection was one of the first analytical techniques used for determining antisense oligonucleotides in biological matrices in support of PK and PK/PD pre-clinical trials.[35-36] The most common isotopes used for this technique include ^{3}H, ^{14}C and ^{125}I.[37-39] The disadvantages associated with radiotracer label methods are low sensitivity, poor selectivity as the degradation products or metabolites cannot be distinguished from the parent, high costs associated with radioisotopes and inability to detect metabolites.

Other inconveniences are problems related to the disposal of radioactive material and the limited use of this technique to pre-clinical trials.[35,40]

High performance liquid chromatography (HPLC)

HPLC is one of the most widely used separation methods due to its availability and robustness. Ion-pair reversed-phase chromatography and strong anion-exchange (SAX) chromatography have been used for the analysis of oligonucleotides.[35] Both are good techniques for the separation of nucleic acids since nucleic acids are charged as a result of the backbone phosphate group. The regular spacing of the phosphate group along the oligonucleotide backbone makes the separation possible based on net negative charge. Unfortunately, poor resolution has been reported with these techniques. Both ultraviolet (UV) and fluorescence detection can be coupled to an HPLC, the sensitivity with fluorescence being greater than that of UV. However, use of either UV or fluorescence detection can only bring the detection limit down to high nanogram levels.[35] Dionex Corporation has introduced a new line of DNAPac® columns (that include guard columns, analytical columns, etc). They claim that DNAPac® columns provide the additional benefit of high resolution with up to single base separation.[41] The sensitivity of the methods using these columns has yet to be determined. As for sample clean-up procedure, a simple liquid-liquid extraction using phenol-chloroform is sufficient when using HPLC type separation.[35]

Liquid chromatography tandem mass spectrometry (LC/MS)

There is currently an explosion in the ever expanding field of LC/MS for solving many current analytical problems. LC/MS is a more attractive alternative to traditional analytical separation techniques considering it offers such features as enhanced sensitivity and performance, speed, high throughput and reliability. The use of LC/MS for the analysis of oligonucleotides has not been very successful due to the multiple charge states that oligonucleotides form in an electro-spray. This has prohibited its use for sensitive quantitation analysis. One key disadvantage of LC/MS is the high price of the instrumentation. On the other hand, LC/MS has been shown to be a useful tool for metabolite characterization.[35]

Hybridization-based enzyme-linked immunosorbent assays

Hybridization assays provide great sensitivity with reported lower limits of quantitation on the order of low pg/mL with high sample throughput using minimal sample clean-up.[42] Reagent and instrument costs for this type of analysis is very low, considering the most expensive piece of equipment is a UV or fluorescence plate reader. The major disadvantage of hybridization based assays is their inability to distinguish an oligonucleotide from its metabolites that may result in cross-reactivity of the metabolites during the quantitation of the parent compound and could result in over-estimation of the parent.

Capillary gel electrophoresis (CGE)

Capillary gel electrophoresis is a fairly recent separation tool. CGE is a powerful method of separating DNA and oligonucleotides and has become the workhorse method of all

separations based genomic studies *e.g.* Human Genome Project.[43] CGE is a powerful technique for separating oligonucleotides since it can be coupled to either a UV or fluorescence detection system. Although very selective, separation of intact oligonucleotides as well as related metabolites that differ by only one or two base units are readily accomplished, CGE-UV is not as sensitive as other analytical techniques such as hybridization assays. Previously validated methods for the quantitation of therapeutic ss-oligonucleotides using CGE UV at 260 nm have demonstrated lower limits of quantitation of ~ 70 ng/mL in plasma and urine.[35-44] A disadvantage of CGE is that the sample clean-up procedure is labour intensive. Samples need to be rid of any proteins, lipids and salts that might potentially interfere with the analysis of the compounds of interest.[8] Since fluorescence is a more powerful detection mode than UV, coupling CGE to LIF could potentially solve the sensitivity issues related to CGE-UV while also alleviating some of the sample preparation constraints.

1.4 Introduction to Capillary electrophoresis

1.4.1 Historical review

Capillary electrophoresis (CE) can be described as a high efficiency separation of small and large molecules in a narrow bore capillary tube filled with an electrolyte buffer solution.[45] The popularity of this relatively new separation technique stems from its ability to separate complex mixtures (proteins, carbohydrates, nucleic acids, various organic and inorganic compounds, *etc.*) with short run times and high separation efficiencies, while requiring only nanolitre amounts of sample.[46] The foundation for CE was laid by Hjerten in the late 1960s when he published his pioneering experiments of

the electrophoretic, free zone separation, using a variety of analytes, in a rotating, millimeter-scale tube.[47] The rotating capillary was immersed in a cooling bath to dissipate the heat generated from the electric current within the tube (also called Joule heating). Mikkers *et al.* revived the technique in the late 1970's when they performed electrophoretic separations using a capillary of smaller diameter that would ultimately help in dissipating the Joule heating more efficiently.[48] The work lead by Jorgenson and Lukacs in the 1980's is considered particularly important since "they treated electrophoresis as an instrumental technique and, as such, introduced capillary electrophoresis to the analytical chemistry community".[43, 49]

1.4.2 Basic configuration

The basic instrumental set-up for a CE system, illustrated in Figure 1-8, consists of a fused silica capillary with an optical viewing window, a controllable high voltage power supply, two electrodes (made of an inert material such as platinum) and an optical viewing window which is aligned with the detector. The ends of the capillary are placed in the electrolyte buffer reservoirs, each containing an electrode connected to a high-voltage power supply. One electrode is connected to the high-voltage output, whereas the other is connected to a return (grounded) cable. After filling the capillary with electrolyte buffer, the sample can be introduced by dipping the inlet end of the capillary into the sample solution and injecting the sample electrokinetically or by hydrostatically.

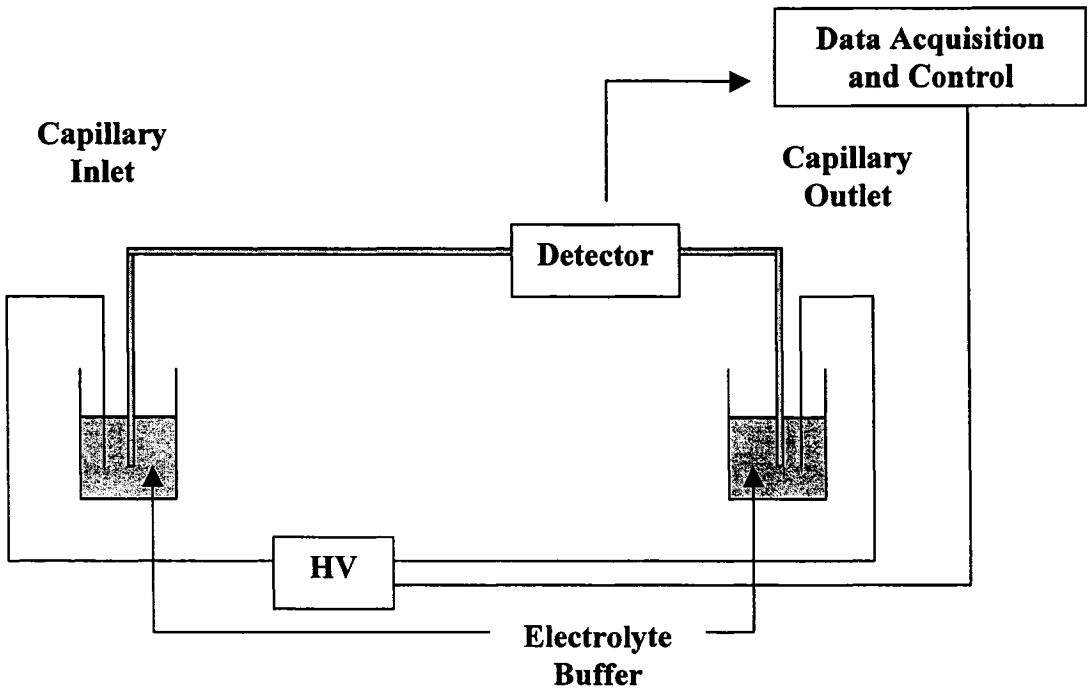


Figure 1-8: Basic instrumental set-up of a CE system is drawn which consists of a fused silica capillary with an optical viewing window, a controllable high voltage power supply, two electrodes and an optical viewing window which is aligned with the detector.

1.4.3 Basic theory and fundamental terminology

The process of electrophoresis is defined as the movement of electrically charged particles or molecules through a conductive fluid or gel under the action of an electromotive force applied to electrodes.[50] If a sample containing a mixture of positive, negative and neutral compounds were electrophoresed they would all migrate at different rates and in different directions, depending on their charge and size. If no other factors are considered, positively charged compounds would move towards the negative electrode (cathode), negatively charged molecules would move towards the positively charged electrode (anode) and neutral molecules would not be influenced by the electric field.

Electrophoretic velocity, electrophoretic mobility and electric field strength

Electrophoresis is the movement of ions under the influence of an applied voltage. The mobility of an ion is governed by its size and ionic charge (mass /charge ratio), therefore smaller ions will move faster than larger ions with the same charge. Electrophoresis relies on differences in the electrophoretic velocity (v_{ep}) of analytes, that in turn, is proportional to the electrophoretic mobility (μ_{ep}) and the electric field strength (E). The relationship between these terms can be summarized by Equation 1-1.

$$v_{ep} = \mu_{ep} E$$

Equation 1-1: Apparent mobility

In capillary electrophoresis, separation is achieved because solutes migrate through the capillary at different velocities. The electrophoretic mobility is a factor that indicates how fast an analyte will migrate through a given medium (electrolyte buffer, gel, *etc.*). It is measured in $m^2V^{-1}s^{-1}$. Electrophoretic mobility is dependent on the volume, charge and shape of the molecule. Thus, small, spherical, highly charged molecules will migrate though a buffer filled capillary the fastest. The electric field strength is a function of the applied voltage divided by the total length of the capillary and is measured in Vm^{-1}.

Electroosmotic flow

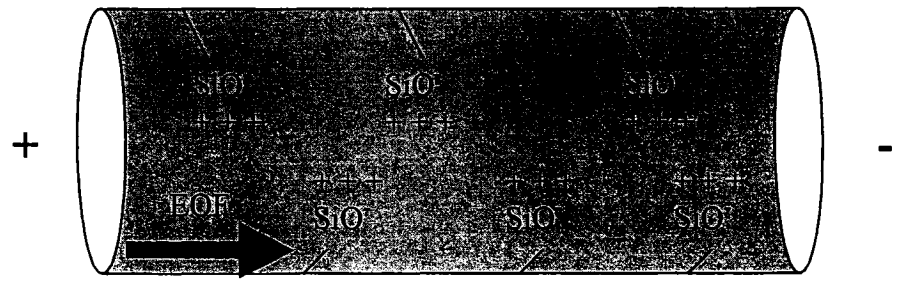
Equation 1-1 is only useful for determining the "apparent mobility". To calculate the actual mobility of an analyte, one must take into consideration the phenomenon of electroosmotic flow (EOF) or electroosmosis. A typical CE separation is performed using a fused silica capillary that has ionizable silanol groups (exposed SiOH with a pka of ~ 1.5) in contact with the buffer (Figure 1-9). Under most CE conditions the capillary wall will be negatively charged, thus attracting positively charged ions from the buffer that will create an electric double layer. When a voltage is applied across the capillary, cations in the diffuse layer will migrate towards the cathode carrying the bulk of the buffer solution with them and resulting in a net flow towards the cathode. A key feature of the EOF is that its magnitude is considerably larger than the electrophoretic mobility. When samples are introduced at the anode end of the capillary, analytes will migrate from the anode towards the cathode, with positively charged analytes moving the fastest, followed by neutral ones and negatively charged analytes migrating the slowest. Consequently, Equation 1-1 should be updated to include the effects on mobility due to EOF (Equation 1-2).

$$v_{actual} = (\mu_{ep} + \mu_{EOF})\, E$$

Where:
μ_{ep} is the electrophoretic mobility
μ_{EOF} is the mobility due to electroosmosis

Equation 1-2: Actual mobility

A) Fused silica capillary under high pH condition: EOF very high



B) Fused silica capillary under low pH condition: EOF reduced

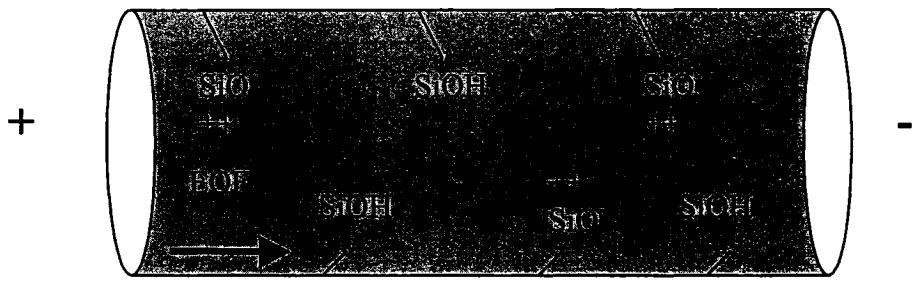


Figure 1-9: Effect of pH on EOF. A typical CE separation is performed using a fused silica capillary that has ionizable silanol groups (exposed SiOH with a pka of ~ 1.5) in contact with the buffer. A) At very low pHs, the magnitude of EOF is large since almost all the exposed silanol groups are negatively charged. B) At higher pHs, EOF is reduced since less silanols are ionized.

EOF can be greatly reduced or even suppressed by coating or even covalently bonding a neutral polymer (e.g.: polyacrylamide) to the inner walls of the capillary. The polymer will shield the charges on the inner surface of the capillary that in turn will reduce or suppress EOF. Once EOF has been eliminated, the rates at which the analytes migrate

through he capillary are solely based on their electrophoretic mobilities and not a combination of electrophoretic mobility and electroosmotic flow.

Migration Time

Another fundamental term in CE is the migration time (MT) of the analyte that is analogous to the retention time in HPLC. MT is the time it takes for the solute to move from the beginning of the capillary the detection window. The migration time is defined by Equation 1-3.

$$MT = L / v_{ep}$$

Where
L is the length of the capillary
v_{ep} is the electrophoretic velocity

Equation 1-3: Migration time

Electropherogram

The data generated in capillary electrophoresis is presented in the form of an electropherogram. An electropherogram is a plot of the migration time versus the detector response.

One of the major advantages associated with capillary electrophoresis is the relatively small sample volumes required for analysis (injection volumes are in the order of nanolitres); however, because capillaries used in CE are anywhere from 50 to 100 micron in internal diameter, the technique can suffer from low sensitivity due to its short pathlength at the detection window.

Detection

The most widely used modes of detection for capillary electrophoresis are ultraviolet/visible (UV/vis) absorbance, fluorescence, mass spectrometry, amperometry and conductivity, however the focus here will be UV/vis and fluorescence.

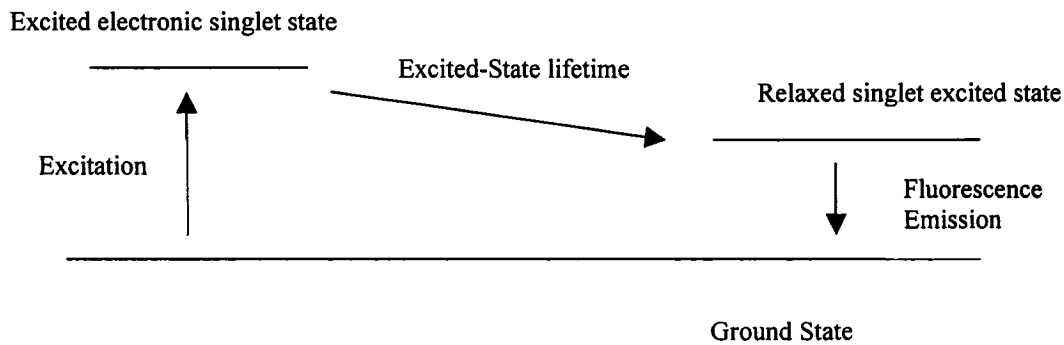
In UV/vis, light hits the capillary, the sample absorbs some of the light and the remaining is transmitted and detected by the photodetector. The principle of UV/vis absorbance (A) is based on Beer's law (Equation 1-4).

$$A = abC$$

Where:
a is the extinction coefficient
b is the path length of the of light in the sample compartment
C is the concentration of the sample

Equation 1-4: Beer's law

Florescence is the result of a three-stage process (excitation, excited-state lifetime and fluorescence emission). Fluorescence occurs when an electron drops from an excited singlet state to the ground state. The excitation energy has a longer wavelength than the emission wavelength.



With fluorescence, detection limits one to three orders of magnitude lower than absorbance detection can be obtained making it a more sensitive detection mode than absorbance. This can be explained by the fact that in absorbance, analytes absorb some of the surrounding light causing a small decrease in light whereas in fluorescence, an analyte produces light in the dark. The intensity of fluorescence detection is proportional to the intensity of the emitted light. With LIF, in particular, very high sensitivity can be achieved because of the high intensity of the incident light and the ability to focus the capillary. Laser-Induced fluorescence can provide sensitivity of up to six orders of magnitude higher than UV absorbance.[50]

Efficiency

The efficiency of a system, which is related to how narrow the peaks are in an electropherogram or chromatogram, and can be expressed as the number of theoretical plates, *N*. The efficiency can be measured using Equation 1-5.

$$N = 16\,(t/w)^2$$

Where
t is the migration time
w is the peak width measured at the base of the peak

Equation 1-5: Efficiency

The narrower the peaks, the higher the efficiency and the better the separation. Most sources of bandbroadening that occur in conventional electrophoresis and/or HPLC are minimized in capillary electrophoresis because of the capillary format employed.

Joule heating, the heat generated within the capillary during the voltage application, is effectively dissipated through the capillary walls, which reduces convection-related bandbroadening encountered in conventional electrophoresis.

Post-separation bandbroadening, related to tubing which connect the separation column to the detector, are eliminated since a portion of the capillary is used as the optical viewing window of the detector.

In systems that require pumps, such as HPLC, laminar flow is observed. In CE, the flow profile, governed mainly by EOF, is plug-like which minimizes sample dispersion during the separation along the capillary.

The major source of bandbroadening in CE is molecular diffusion. Large molecules, such as proteins, have smaller diffusion coefficients than smaller molecules. Consequently, the dispersive effect of the larger molecules on peak broadening will be lower.

Capillary Gel Electrophoresis

Different modes of capillary electrophoresis are available, such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP) and capillary gel electrophoresis (CGE).

Due to similarities in chemical properties between the four nucleotides, when a single base is added to a strand of oligonucleotides, it changes the size as well as the charge of the molecule proportionally, so the size to charge ratio will remain about the same. Since the size to charge ratio does not vary much, oligonucleotides or DNA fragments are not

well separated by capillary zone electrophoresis, however they can be by CGE. The ideal separation mode for DNA is CGE.

CGE is a rapidly developing method for separation of biopolymers. CGE is performed in a capillary filled with a network of UV transparent polymer, most frequently crosslinked gels or entangled linear polymers that acts as a molecular sieve. This process is analogous to traditional SDS-PAGE. Initially, crosslinked rigid gels were used. More recently, introduction of entangled polymers has allowed easy filling and flushing of the capillary after each run without any significant loss of resolution. The gel serves two purposes. First, it serves to diffuse convective currents that would result in localized heating in the matrix that would result in irregular migration patterns. Second, the gel also creates a molecular sieve that enhances the separation based on molecular weight. In this gel, DNA strands can be separated according to both their electric charge (as with all electrophoresis) and their size, with large strands taking longer to pass through the pores in the gel than smaller strands. By varying the pore size, very sensitive separations can be achieved, such that DNA strands differing by only a single base can be individually resolved.[50]

1.5 Oligonucleotides and Fluorescence

Only certain molecules which contain polyaromatic hydrocarbons or heterocycles will fluorescence. These molecules are given the name of fluorophores. Nucleic acids are not strong fluorophores (refer to the structure of nucleic acids Figure 1-2 and Figure **1-3**). Because nucleic acids do not possess strong fluorescing properties, they require probes or dyes that will bind to a string of nucleic acids (oligonucleotide) and fluoresce.

Derivatization can be by covalently binding a fluorescent tag to the oligonucleotide, for

example fluorescein isothiocyanate, or by staining a sample of oligonucleotides with dyes which form non-covalent bonds with its target, for example OliGreen™, PicoGreen™, SyberGreen™, RiboGreen™, *etc.*

1.6 Thesis Organization

The goal of the work described here was, based on the knowledge acquired about capillary gel electrophoresis and antisense oligonucleotides, to develop and validate a method for the quantitative determination of single-stranded antisense oligonuceleotides in plasma using capillary gel electrophoresis using LIF detection. Since CGE already has the desired specificity for quantifying ss-ASO, this novel method should ideally enhance the sensitivity over that seen with CGE-UV (reported lower limit of quantification in the literature = 70 ng/mL).[12]

Chapter 2 of the thesis details the methodology for optimization of the procedure as well as the final conditions of the capillary electrophoresis system.

Chapter 3 describes the validation of the final optimized method to industry standards. The validation parameters that were looked at were selectivity/specificity (which, as per the FDA, can be used interchangeably), carry-over, linearity (or range of linear response), intra- and inter-assay precision and accuracy.

And the final chapter, Chapter 4, summarizes the results presented in the first two chapters and presents suggestions for future work.

Chapter 2

Method Development / Optimization

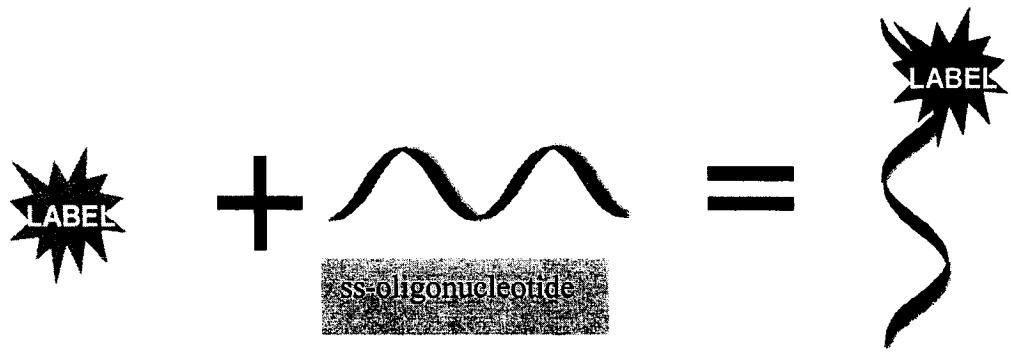
2.1 Introduction

Capillary gel electrophoresis is one of the preferred analytical techniques for the determination of therapeutic oligonucleotides. It separates molecules as they move through a polymer network, which acts as a molecular sieve. As the analytes migrate through the sieve, they become hindered, with large DNA molecules more hindered than smaller ones.

In previously published studies, methods utilizing CGE with ultraviolet (UV) detection have been developed for the quantitative determination of oligonucleotides in various matrices of biological importance *e.g.* plasma, serum and tissues. While very selective (separation of intact oligonucleotides as well as related metabolites that differ by only one or two base units), CGE-UV is not very sensitive in comparison to other analytical techniques *e.g.* hybridization assays, for quantifying oligonucleotides. Previously validated methods for the quantitation of oligonucleotides using CGE-UV have demonstrated a lower limit of quantitation (LLOQ) of approximately 70 ng/mL. Recently, LIF detection for CGE has emerged as an attractive alternative to UV detection, that may significantly improve the sensitivity of CGE.

Oligonucleotides do not possess significant native fluorescence, therefore the use of LIF would require a molecule capable of forming a stable fluorescent complex with the ss-oligonucleotide. The unbound (free) fluorescent molecule would need to have low

fluorescence, however fluorescence enhancement would be required upon binding to the ss-oligonucleotide so as to have minimal background noise.



In 1996, Reyderman and Stavchansky published an article in the Journal of Analytical Chemistry that described a method for determining single-stranded oligonucleotides using CGE-LIF.[51] The method utilized a nucleic acid stain called OliGreen™, manufactured by Molecular Probes and achieved a lower limit of quantitation (LLOQ) of 1 ng/mL.

The initial CGE conditions for method development were based on the Reyderman and Stavchansky article, however several important modifications had to be made for a successful separation. The sample pre-treatment methodology was based on the article of Leeds and al.[8]

As part of method development, parameters such as optimal dye concentration, optimal dye choice and capillary temperature were determined and results will be discussed in this section.

2.2 Experimental Procedure

2.2.1 Materials and Reagents

Fused silica capillaries (BioCap linear polyacrylemide (LPA) coated capillary) were obtained from Bio-Rad (Philadelphia, PA). ssDNA 100-R (replaceable) gel capillary kit and the 100 mM Tris borate buffer with 7 M urea were obtained from Beckman Coulter (Montreal, Que). The replaceable gel and buffers were prepared according to the instructions in the package inserts. Strong anion-exchange (SAX) and C_{18} 96-well extraction plates were purchased from Phenomenex (Montreal, Que). Human plasma (pool of male and female and EDTA as anticoagulant) was purchased from Bioreclamation (Montreal, Que).

Acrylamide, Tris, ammonium persulfate, N,N,N',N'-Tetramethylethylenediamine (TEMED, urea and ((Methacryloxy)propyl)trimethoxysilane were obtained from Sigma (Montreal, Que).

Reagent grade methanol and acetonitrile were purchased from JT Baker (Montreal Que). Filters (0.2 μm) were purchased from Baxter. Drop-dialysis cellulose acetate membranes of 0.025 micron pore size were purchased from Millipore (Cambridge, Ont). OliGreen™, PicoGreen, RiboGreen, SyberGreen I and II and SyberGold were obtained from Molecular Probes Inc. (Eugene, Oregon).

27- and 31-mer poly(dT), were purchased from Synthegen. The oligonucleotides used in the final stages of method development and for the actual validation of the method were single-stranded phosphothioate oligonucleotides (24-mer parent, 23 (n-1), 22-mer (n-2)

and 18-mer internal standard). Considering that this project was a joint collaboration between Concordia University and Charles River Laboratories Preclinical Services Montreal, the sequence of the oligonucleotide cannot be revealed at this time for confidentiality reasons, however the oligonucleotides were a mixture of the four bases A, T, G and C.

2.2.2 Apparatus

All electrophoretic separations were carried out on a Beckman P/ACE MDQ System equipped with an argon-ion laser detector (Laser Module 488, Beckman) and a 48-position auto-sampler.

2.2.3 Standards

The 27-mer and 31-mer poly(dT) phosphothioate antisense oligonucleotide were received in powder format and reconstituted using ultra pure water. Standards were prepared plasma using EDTA as anticoagulant.

2.2.4 Summary of Capillary Electrophoresis Conditions:

The standard, QC and blank sample extracts were analyzed by CGE using the following conditions:

CE System:	Beckman P/ACE System MDQ
Capillary:	BioCap LPA (31 cm x 75 μm id; 21 cm effective length)
Gel type:	ssDNA 100-R gel
Capillary temperature:	variable for method development
Running buffer:	100 mM Tris borate, 7 M urea buffer solution

Injection conditions:	electrokinetic
	10 kilovolts (reverse polarity - cathode in sample vial)
	2 to 20 seconds
Separation conditions:	20 kilovolts (reverse polarity) - ramped at 0.17 min
	15 psi applied to both ends of the capillary
LIF detection (argon-ion):	excitation wavelength: 488 nm
	emission wavelength: 520 nm
Run time:	variable

The LPA coated capillaries were first rinsed with ultra pure water and the running buffer. Next, the capillaries were filled with the sieving matrix (ssDNA 100-R gel) followed by a conditioning step with the fluorogenic dye.

Following every ~10 injections, the gel inside the capillary was rinsed out and the capillary replenished with fresh gel. The capillary was rinsed three times with buffer before each gel fill to ensure that no dye is left in the capillary which would negatively impact the following next injection.

Preliminary tests using OliGreen™ indicated that the background signal, without any dye present, was close to 0 relative fluorescence units (RFUs). However, once the dye reached the detection window, an increase in fluorescence could be observed. This increase was subsequently used as the primary indicator that the dye had migrated and reached the detection window which in turn signified that the capillary was saturated with the dye and fully conditioned (the initiation of the dye front can be translated into the

migration time of the dye). Note that it is not uncommon in capillary electrophoresis to observe unknown peaks that are unrelated to the analyte and randomly appear in an electropherogram and will be termed "ghost peaks".

2.2.5 Data Collection and Statistical Analysis of Data

Data collection was performed using 32 Karat Software, version 5.0, from Beckman Coulter (Montreal, Que).

2.3 Method Development

2.3.1 Dye concentration

The first step in optimizing dye concentration was to determine the ideal dilution factor. Reyderman and Stavchansky[51] used dilution factors of 200 and 400 for OliGreen™, with the 200 fold dilution giving better results. We decided to verify this in our laboratory. The dye was diluted using the running buffer. We decided to expand on this range by diluting from 1:150 to 1:1500 with the running buffer.

2.3.2 Dye Choice

Molecular Probes have designed a variety of commercially available nucleic acid stains that are for ultrasensitive nucleic acid quantitation. These patented nucleic acid-binding stains are symmetric cyanine dimer derivatives and share several unique and outstanding properties[52], such as:

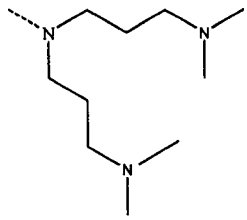
- High molar absorptivity, with extinction coefficients typically greater than 50,000 $cm^{-1}M^{-1}$ at visible wavelengths.

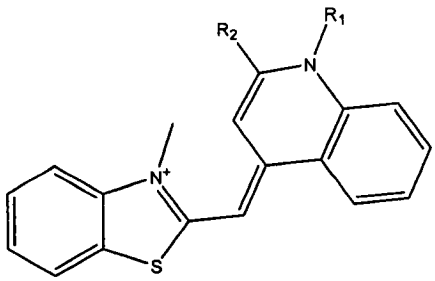
- Very low intrinsic fluorescence, with quantum yields usually less than 0.01 when not bound to nucleic acids.
- Large fluorescence enhancements (often over 1000-fold) upon binding to nucleic acids, with increases in quantum yields to as high as 0.9
- Moderate to very high affinity for nucleic acids, with little or no staining of other biopolymers.

The cyanine dyes show differences in some physical characteristics, such as nucleic acid specificity, that allow their distinction. For the analysis of nucleic acids, Molecular Probes recommends to use the following cyanine based dyes: PicoGreen™, OliGreen™ and RiboGreen™, SYBR Gold™, SYBR Green I™ and SYBR Green II™, SYBR Safe DNA gel stain™, SYBR DX DNA™ blot stain and CyQUANT GR™ dye. From this list of suggested dyes, six were tested in our laboratory for detecting single-stranded oligonucleotides (Table 2-1). All of the dyes were supplied in a dimethylsulfoxide (DMSO) solution.

These dyes have the same cyanine core however modifying their side groups significantly alters their physical properties *e.g.* changing the aromatic rings, the number of carbon atoms linking the cyanine core and the side groups *etc.* The structures of SyberGreen I™ and PicoGreen™ are depicted in Figure 2-1.[53]

For SyberGreen R_2 =





Cyanine dyes where R_1 = Phenyl

For PicoGreen R_2 =

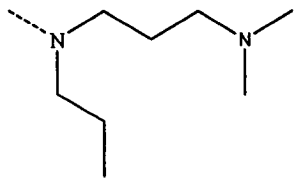


Figure 2-1: Chemical structure of dyes. The dyes have the same cyanine core however with modified side groups.

As indicated above, six nucleic acid stains provided by Molecular Probes were tested in our laboratory for detecting single-stranded oligonucleotides. Unless otherwise noted, a dilution factor of 500 fold was performed with the dyes.

Table 2-1: Nucleic acid stains

Dye	Target	λ_{ex} (nm)	λ_{em} (nm)	Quantum Yield (QY)
PicoGreen	Double-stranded DNA	502	523	0.01
RyboGreen	RNA	500	523	0.01
OliGreen	ss-oligonucleotides	500	523	0.01
Syber Dyes • SYBR Green I • SYBR Green II • SYBR Gold	Double-stranded and single stranded DNA in electrophoretic gels	300/500	537	0.01

2.3.3 Temperature effect

As discussed in previous sections, the resolution and peak shape between the oligomers required improvement. The final and most important variable to consider was the temperature of the capillary. A 27-mer and 31-mer poly-dT oligonucleotide was injected at a concentration of 1 microgram/mL. The remaining separation conditions were kept constant. First, injections of the oligomer solution was done with the capillary separation temperature set at 30°C followed by separation temperatures of 40, 45, 50 and 60°C. Higher separation temperature could not be used since 60°C was the upper limit allowed by the instrument.

2.4 Results and Discussion

2.4.1 Dye concentration

As mentioned above, method development was initiated using OliGreen™, which was based on the published work by Reyderman and Stavchansky[51]. The migration time of the dye was inconsistent between dye conditionings as well as between capillaries. This may be a result of the aging of the capillary coating. In theory, coated capillaries should either greatly reduce or even suppress EOF. However, the aging process might be deteriorating the inner capillary wall coating, which would expose ionisable silanol groups, and, in turn, increase EOF. It was also observed that within the same day, when using the same diluted dye vial, the migration time of the dye (dye conditioning) increased from injection to injection. This may be due to natural decay through oxidation.

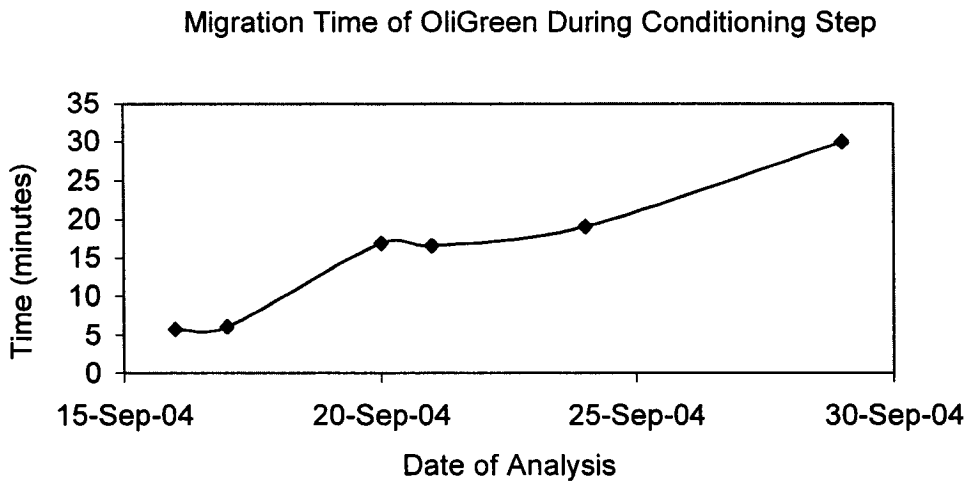


Figure 2-2: Inconsistencies between OliGreen™ conditionings is depicted. The same capillary was used for this experiment. Conditioning step was performed with every new gel fill. Migration time of dye increases with time which may be due to the aging of the capillary wall coating.

Standards containing both the 27-mer and 31-mer oligonucleotides were prepared at 0.3 μg/mL and were injected immediately following the dye conditioning step. The resulting electropherograms showed large peaks and therefore confirmed that the dye was interacting with the oligonucleotides. The two oligomers were not resolved, however for method development purposes, the fact that large peaks were observed was a clear indication of sensitivity enhancement.

With a dilution of 150 fold, the fluorescence signal was high, however the two oligomers were not well resolved and splitting of the peaks was observed. This suggests that the dye-oligonucleotide complex may be forming secondary, tertiary or quaternary structures which, in turn, affect the migration time thus causing the split peaks. With a dilution of 700 fold, the fluorescence signal was lower than that with a 150 fold dilution, however the resolution between the two oligomers improved and no splitting of the peaks was observed. With a 500 fold dilution factor, following passing of the oligonucleotide from the detection window, relative to the peak height, a slight dip in fluorescence, below the baseline, was observed. This can be explained by a depletion of the dye in that area since most of the dye complexes with the oligonucelotide. With a 1500 fold dilution, the fluorescence signal is the lowest. The 27- and 31-mer oligomers were well resolved as well as the degradation products. Also, no splitting of the peaks can be observed. However, considering the low signal with the 1500 fold dilution, the dip in fluorescence following the oligonucleotide peaks are more significant.

Considering factors such as minimum conditioning time of the capillary, high fluorescence enhancement and good resolution, it was decided to use a 500 fold dilution for future analysis.

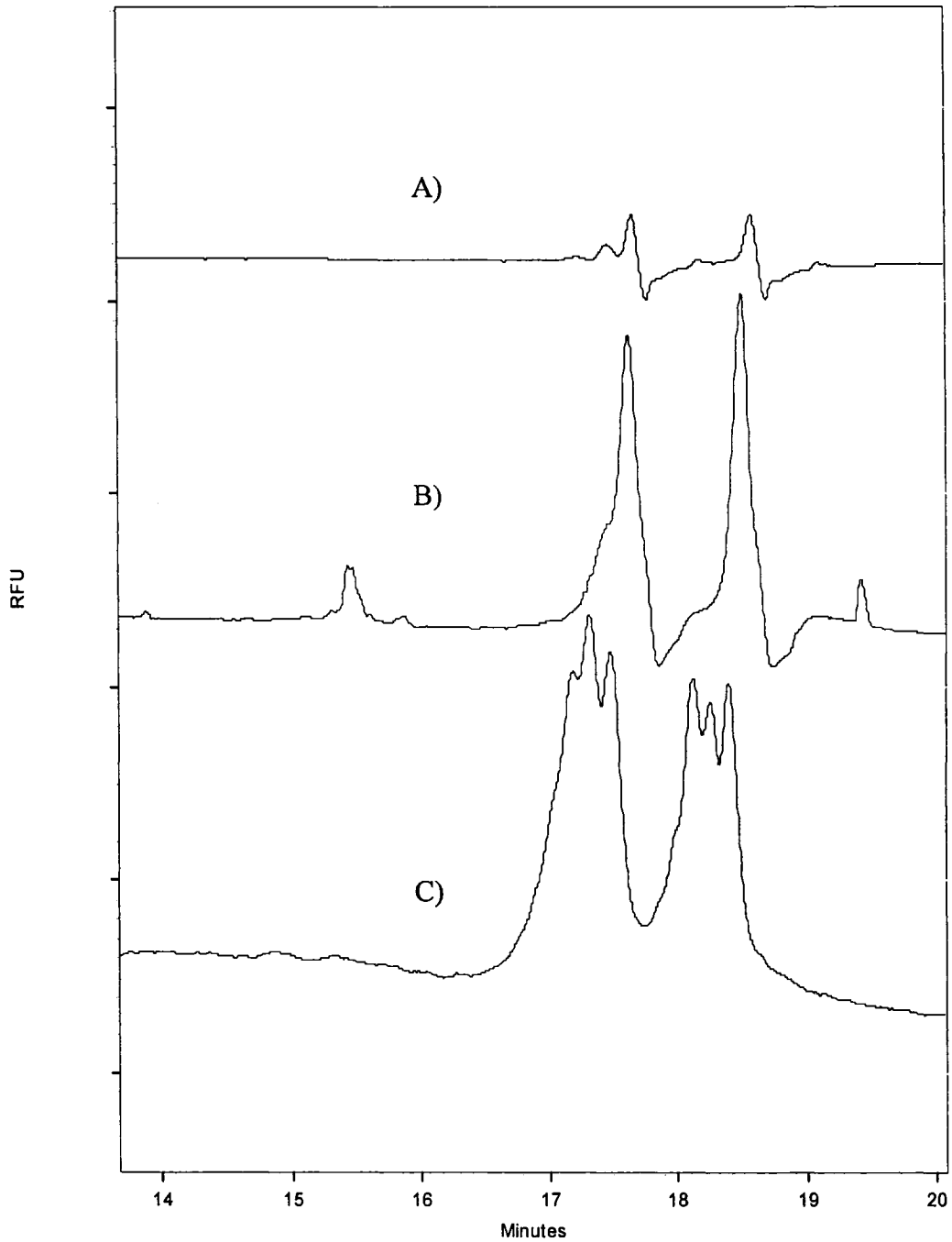


Figure 2-3: Effect of Dye concentration on peak shape (traces have been moved on Y-axis). A) 1500 fold DF was applied. The response was lowest (~ 0.65 RFUs), the two oligomers were resolved and no peak splitting observed. B) 500 fold DF was applied. The response was lower (~ 4 RFUs), the two oligomers were resolved and no peak splitting observed. C) 150 fold dilution factor (DF) was applied. Electropherogram showed high fluorescence response (~ 13 RFUs), 27 and 31-mer oligomers were not resolved and peak splitting observed.

2.4.2 Dye Choice

Unless otherwise noted, a dilution factor of 500 fold was used for the dyes.

OliGreen™

OliGreen™ was the first tested nucleic acid stain since there was published data indicating that this dye interacts well with short single-stranded DNA. Since OliGreen™ is complexed with a ss-oligonucleotide, it was assumed that the interaction between the dye and the oligonucleotide is an electrostatic one. Following the conditioning of the capillary with OliGreen™, an increase in fluorescence was observed. The conditioning step varied from a minimum of 12 minutes to a maximum of 30 minutes. The dye front was, on average, approximately 2 RFUs. Standards containing both the 27-mer and 31-mer oligonucleotides were prepared at different concentrations and injected immediately following the dye conditioning step. The peaks were distorted and not well resolved. The first injection showed relatively high fluorescence and relatively good resolution. However, the fluorescence of the subsequent injections, in comparison to the previous injection, was to decreased by almost half. Also, the peaks for the 27-mer and 31-mer oligonucleotides for all subsequent injections in this preliminary work were not well resolved.

PicoGreen™

PicoGreen was tested next. The Product Information for PicoGreen™ claimed high affinity for double-stranded DNA, and therefore it was not clear if it would bind to ss-DNA. Following the conditioning of the capillary with PicoGreen™, a lower increase in fluorescence was observed in comparison to OliGreen™. The dye front was approximately 6 RFUs (Figure 2-4).

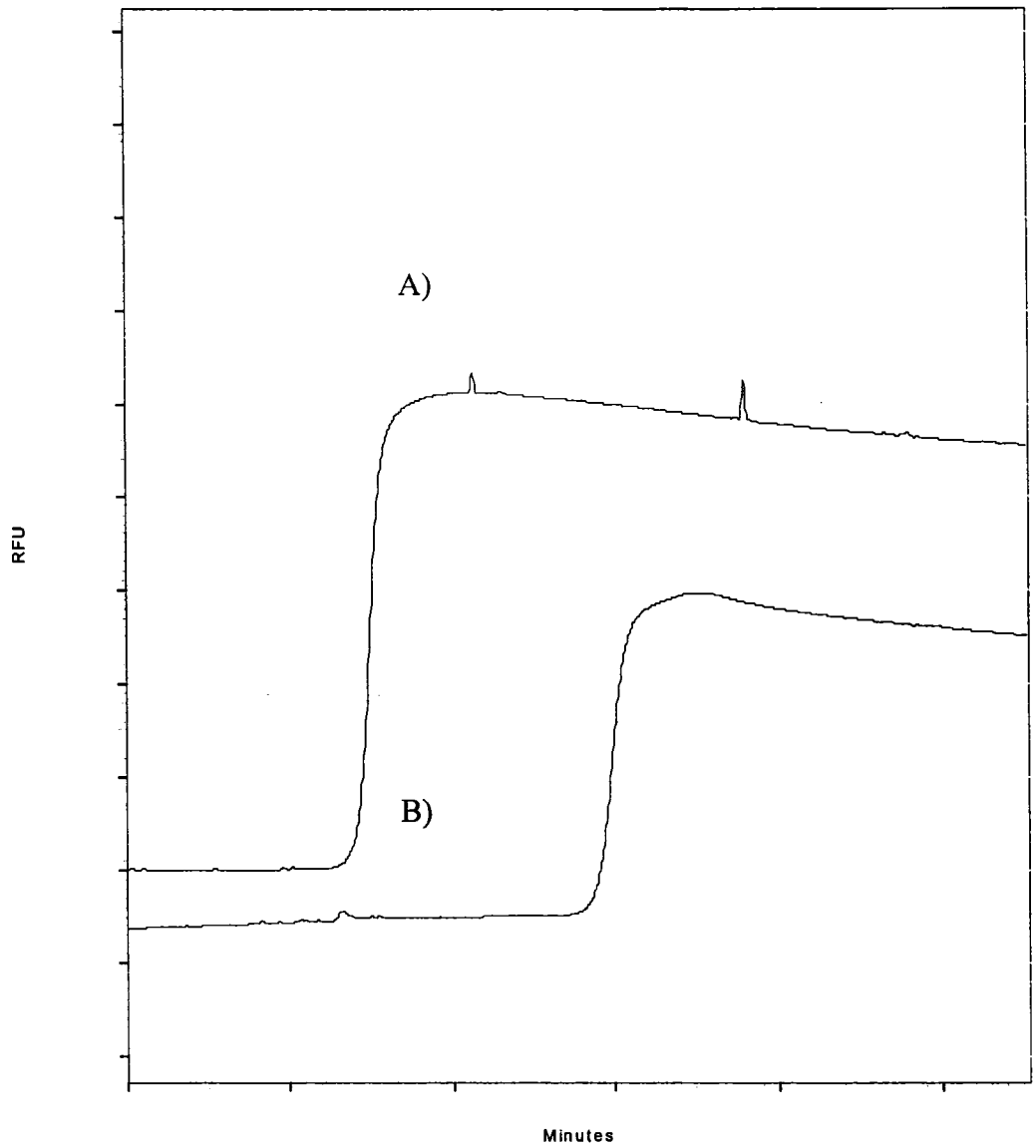


Figure 2-4: Representative electropherograms of dye conditionings with A) OliGreen™ and B) PicoGreen™ (traces have been moved on both the X- and Y-axis). With OliGreen™, the increase in fluorescence is approximately 1.5 times greater than that with PicoGreen

When a standard spiked with a 27- and 31-mer poly(dT) was injected following the conditioning step, peak splitting was observed as well as a significant dip in florescence following the peak for the 31-mer. The response (RFU) was also lower with the use of PicoGreen™. It was thus confirmed that PicoGreen binds to single-stranded DNA, however, OliGreen™ remains a better candidate.

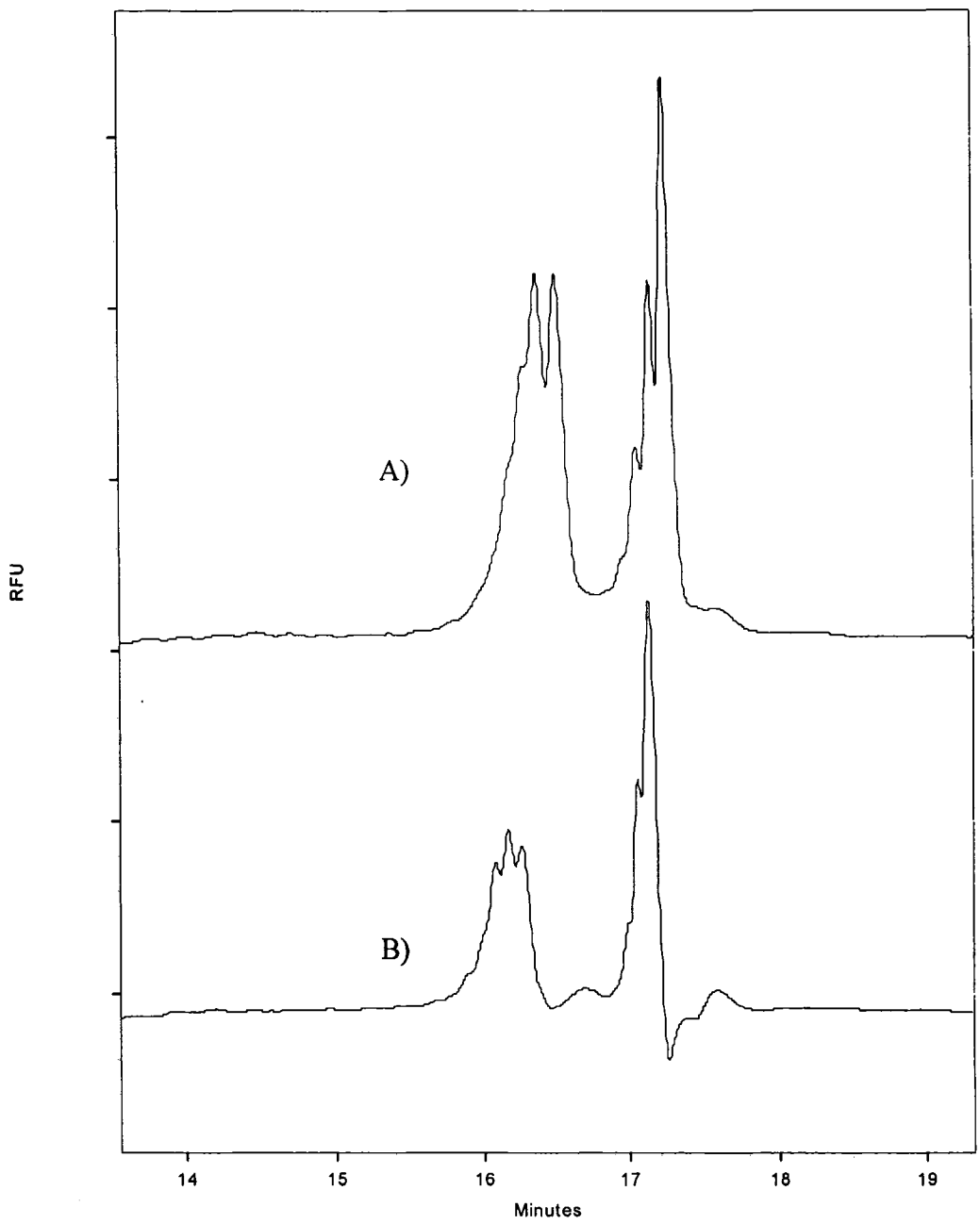


Figure 2-5: Representative electropherograms with A) OliGreen™ and B) PicoGreen™ (traces have been moved on the Y-axis). With OliGreen™, the increase in fluorescence is approximately 1.5 times greater than that with PicoGreen™ (consistent with the dye conditioning step). With PicoGreen™, a dip in fluorescence occurs following the 31-mer oligomer, which would increase the error during quantitation.

SyberGreen I, SyberGeen II and SyberGold

SyberGreen I™, SyberGeen II™ and SyberGold™ all showed an increase in fluorescence, less intense than that with OliGreen™ and PicoGreen™, during the dye conditioning step (Figure 2-6).

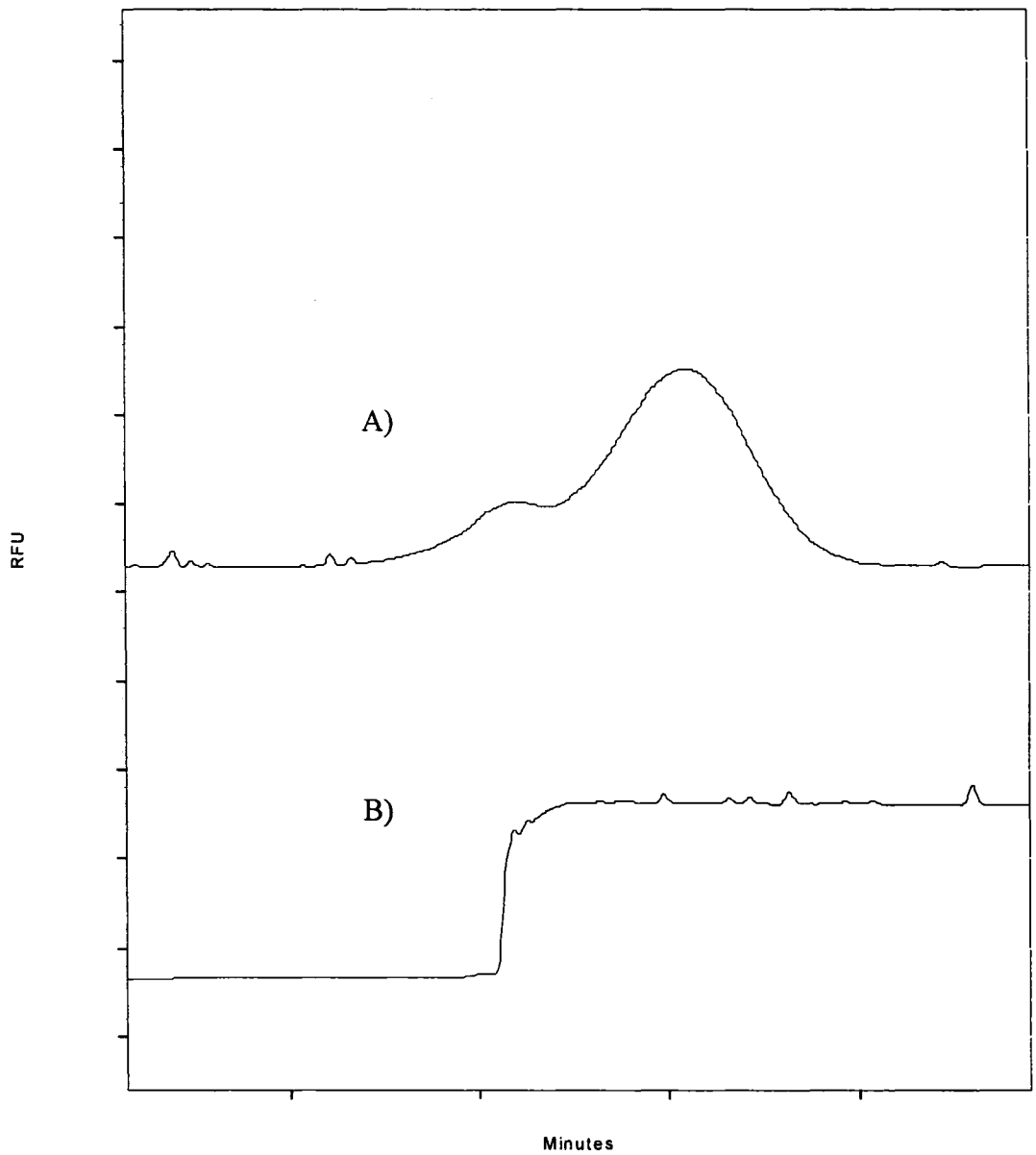


Figure 2-6: Representative electropherogram of (A) ss -oligonucleotide standard injection with the use of SyberGreen I and (B) dye conditioning (traces have been moved on the Y-axis).

However, when a standard was injected immediately following the dye conditioning step, the peaks for the oligonucleotides were distorted and the two oligomers were unresolved (Figure 2-6). The reasons for these results are unknown.

RiboGreen™

The results obtained with RiboGreen™ were very similar to the results obtained with SyberGreen I™, SyberGeen II™ and SyberGold™; an increase in fluorescence, less intense than that with OliGreen™ and PicoGreen™, during the dye conditioning step was observed. However, when a standard was injected immediately following the dye conditioning step, the peak for the oligonucleotides was distorted and unresolved between oligomers.

In conclusion, it was decided to continue the work with OliGreen™, as it was most suitable for detecting short single-stranded DNA and it was previously used for the same type of assay in other laboratories (*e.g.* Ryderman and Stavchansky).

2.4.2.1 Temperature effect

The final and most important variable to consider was the temperature of the capillary. The initial experiments conducted were done using a capillary temperature of 30°C since this was the optimal separation temperature reported by Yu *et al.*[42] It was observed that an increase in capillary temperature showed significant improvements in peak resolution. Peak splitting disappeared which in turn improved the reproducibility and resolution of the separation (Figure 2-7). The best results were obtained with a capillary temperature of 60°C. The melting temperature of DNA ranges from 50 to 70, depending on the sequence, which, in turn, means at higher separation temperatures, self-hybridization of

the short-single stranded oligonucleotides is less likely to occur. This would explain why the best electropherograms (no peak splitting, good resolution) were obtained at 60°C.

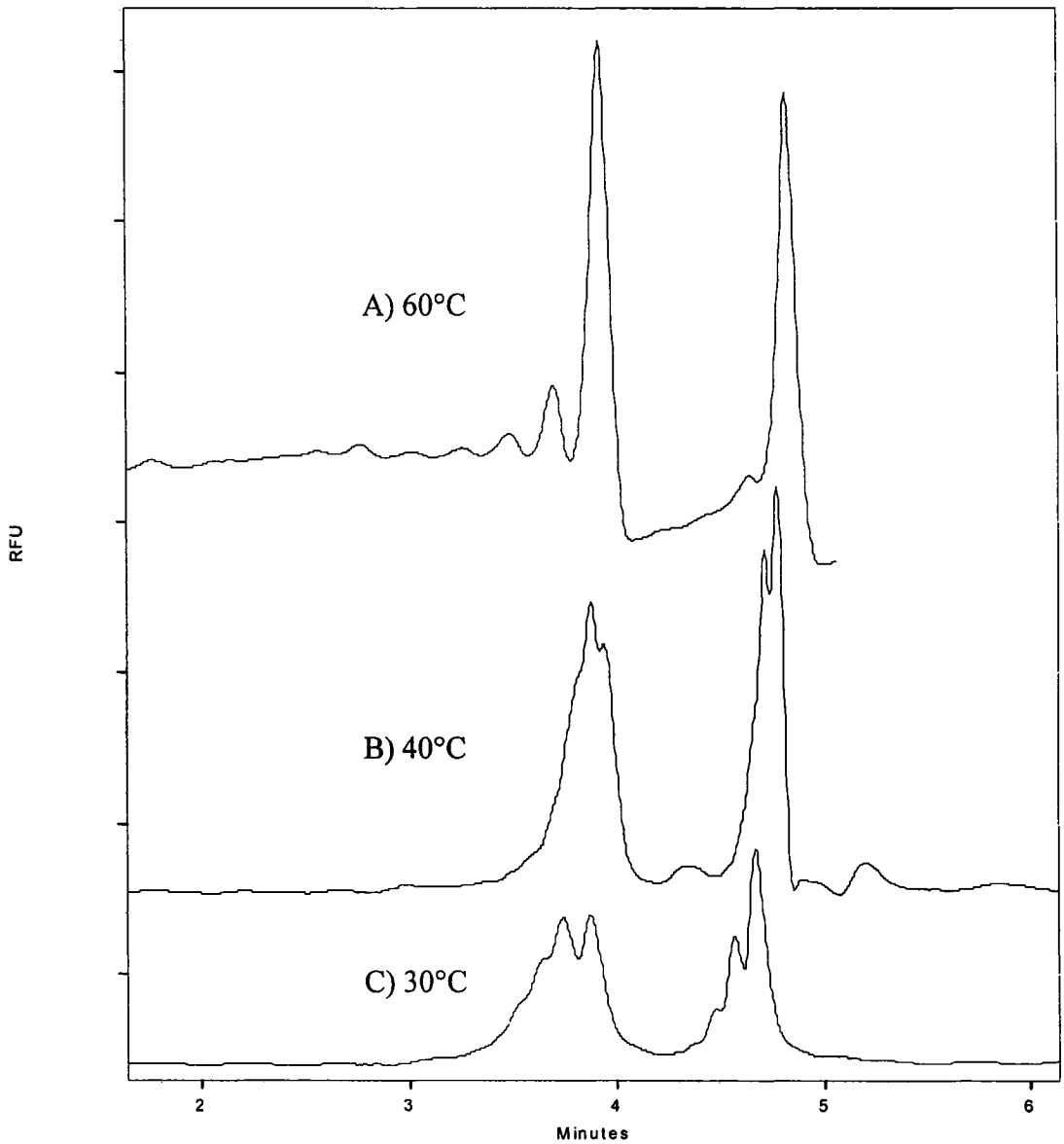


Figure 2-7: Temperature effect on oligonucleotide separation (traces have been moved on Y-axis). A) Capillary temperature was set at 60°C. 27 (MT ~16.8 minutes), 31-mer (MT ~17.5 minutes) as well as degradants are resolved. No peak splitting observed. B) Capillary temperature was set at 40°C. 27 (MT ~15 minutes) and 31-mer (MT ~16 minutes) are resolved. Less peak splitting observed. C) Capillary temperature set at 30°C. 27 (MT ~15.5 minutes) and 31-mer (MT ~16.3 minutes) are resolved, however the individual peaks are split.

2.4.3 Method optimization

To further confirm our findings prior to moving on to the validation, separations, using the settings determined during the development stage (OliGreen™ with a 500 fold dilution factor) were performed using standards spiked with the quasi-random 24-mer phosphotioate oligonucelotide and its n-1 and n-2 metabolites (23- and 22-mer) using an 18-mer oligonucleotide as internal standard (IS). The separations obtained had better resolution and more reproducibility than those obtained with the poly(dT). At the time method development started, Molecular Probes did not offer much information regarding OliGreen™, however our data indicated that the OliGreen™ interacted better with a mixed sequence of oligonucleotides (mixture of A, G, T and C) than with a poly(dT). In 2005, Molecular Probes updated the OliGreen™ product information sheet to indicate that this dye has high affinity for thymine (T). This could explain the observed variability between injections (reproducibility and resolution issues) which would probably be due to a quenching effect.

2.5 Conclusion

In summary, the method development experiments indicated that the ideal nucleic acid stain to use for the quantifying short single-stranded oligonucleotides is OliGreen™. The optimal dilution to perform is a 500 fold dilution. The capillary temperature should be set at 60°C. The capillary needs to be saturated with the dye prior to sample injection. Ideally, a dye conditioning (migration time of the dye to the detection window) which takes between 12 to 30 minutes yields the best reproducibility and resolution for the following injections.

Following every approximately 10 injections, the gel inside the capillary should be rinsed out and the capillary is replenished with fresh gel. To ensure the resolution is not compromised, the capillary requires adequate cleaning with buffer before each gel fill. If any dye is left in the capillary, it will negatively impact the following next injection.

Chapter 3

Method Validation

3.1 Introduction

In Chapter 2, a detailed description of developing and optimizing a method for determining single-stranded oligonucelotides in plasma using capillary gel electrophoresis and LIF was outlined. Chapter 3 will discuss the second phase of the project, which was the validation of the above-mentioned method.

Validation was based on the document "Guidance for Industry, Bioanalytical Method Validation" issued by the "United States Department of Health and Human Services, FDA Center for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM)" in May 2001. The current method was validated for the 24-mer parent oligonucleotide in plasma (EDTA used as anticoagulant) with respect to selectivity, carry-over, linearity, lower limit of quantitation (LLOQ), and intra- and inter-assay precision and accuracy. The data generated for the 23-mer and 22-mer metabolites are for qualitative purposes only since there are no acceptance criteria for metabolites.

3.2 Experimental Procedure

3.2.1 Materials and Reagents

Fused silica capillaries (BioCap linear polyacrylemide (LPA) coated capillary) were obtained from Bio-Rad (Philadelphia, PA). ssDNA 100-R (replaceable) gel capillary kit and the 100 mM Tris borate buffer with 7 M urea were obtained from Beckman Coulter (Montreal, Que). The replaceable gel and buffers were prepared according to the

instructions in the package inserts. Strong anion-exchange (SAX) and C_{18} 96-well extraction plates were purchased from Phenomenex (Montreal, Que). Human plasma (pool of male and female and EDTA as anticoagulant) was purchased from Bioreclamation (Montreal, Que).

Reagent grade methanol and acetonitrile were purchased from JT Baker (Montreal Que). Filters (0.2 μ) were purchased from Baxter. Drop-dialysis cellulose acetate membranes of 0.025 μ pore size were purchased from Millipore (Cambridge, Ont). OliGreen™ was obtained from Molecular Probes Inc. (Eugene, Oragon). The oligonucleotides used for the validation of the method were single-stranded phosphothioate oligonucleotides (24-mer parent, 23 (n-1), 22-mer (n-2) and 18-mer internal standard). Considering that this project was a joint collaboration between Concordia University and Charles River Laboratories Preclinical Services Montreal, the sequence of the oligonucleotide cannot be revealed at this time for confidentiality reasons, however the oligonucleotides were a mixture of the four bases A, T, G and C.

3.2.2 Apparatus

All electrophoretic separations were carried out on a Beckman P/ACE MDQ System equipped with an argon-ion laser detector (Laser Module 488, Beckmann Coulter) and a 48-position auto-sampler.

3.2.3 Standards

Standards of a 24-mer phosphothioate antisense oligonucleotide (parent analyte) and metabolites (n-1, 23-mer and n-2, 22-mer) were prepared in plasma (EDTA was used as the anticoagulant) covering the nominal parent, n-1 and n-2 concentration range of 0.250

to 25.0 ng/mL, containing internal standard (18-mer) at a nominal concentration of 10.0 ng/mL. The volume of plasma used for each analysis was 1 mL.

3.2.4 Quality Control Samples

Quality control (QC) samples of parent and metabolites were prepared in blank plasma (EDTA) at nominal parent, n-1 and n-2 concentrations of 1.00, 1.50, 2.00, 5.00, 15.0 and 20.0 ng/mL containing internal standard (18-mer) at a nominal concentration of 10.0 ng/mL. The volume of plasma used for the analysis was 1 mL.

3.2.5 Analysis

To each standard, QC sample and blank sample, 10 µL of a 1.00 µg/mL internal standard was added. For double blank samples, 10 µL of water was added in place of the internal standard; for single blanks with analyte and metabolites only, no internal standard was added; for single blanks with internal standard only, 10 µl of the 1.00 µg/mL solution was added). The 1.0 mL samples were diluted with 1.5 mL strong anion-exchange (SAX) loading/running buffer (10 mM Tris buffer solution, pH 9.0, containing 0.25 M potassium chloride and 20% acetonitrile (by volume) and vortexed for approximately 2 seconds. The samples were then loaded onto SAX 96 well plates that had been preconditioned with acetonitrile (2 mL), water (2 mL), and SAX loading/running buffer (2 mL). Each well was then washed with 8 mL SAX loading/running buffer, and the analytes eluted with 1.5 mL SAX elution buffer (10 mM Tris, pH 9.0, containing 0.5 M potassium chloride and 1 M sodium bromide and 5% acetonitrile).

The eluate was loaded onto reversed-phase C18 96 well plates that had been preconditioned with 2 mL acetonitrile, 2mL water, and 2 mL reversed-phase dilution

buffer (10 mM Tris buffer solution, pH 9.0, containing 0.5 M potassium chloride and 1 M sodium bromide). Each well was washed with 8 mL water, and the analytes were eluted 1.5 mL 20:80 (v/v) acetonitrile:water. The eluate was evaporated to dryness, the residue re-dissolved in 50 μL water, vortexed for 30 seconds, sonicated for 5 minutes, centrifuged at 10000 rpm, 10°C for 30 seconds, and desalted on a 96 well desalting plate suspended on water for at least 3 hours at room temperature. Note that the extraction procedure described above is based on the article published by ISIS pharmaceuticals with some minor modifications.[8]

3.2.6 Summary of Capillary Electrophoresis Conditions:

The standard, QC and blank sample extracts were analyzed by CGE using the following conditions:

CE System:	Beckman P/ACE System MDQ
Capillary:	BioCap LPA (31 cm x 75 μm id; 21 cm effective)
Gel type:	ssDNA 100-R gel
Capillary temperature:	set at 60°C
Running buffer:	100 mM Tris borate, 7 M urea buffer solution
Injection conditions:	electrokinetic
	-10 kilovolts (reverse polarity - cathode in sample vial)
	2 to 20 seconds
Dye conditioning:	variable (20 to 30 minutes)
Separation conditions:	-20 kilovolts (reverse polarity) – ramped at 0.17 min

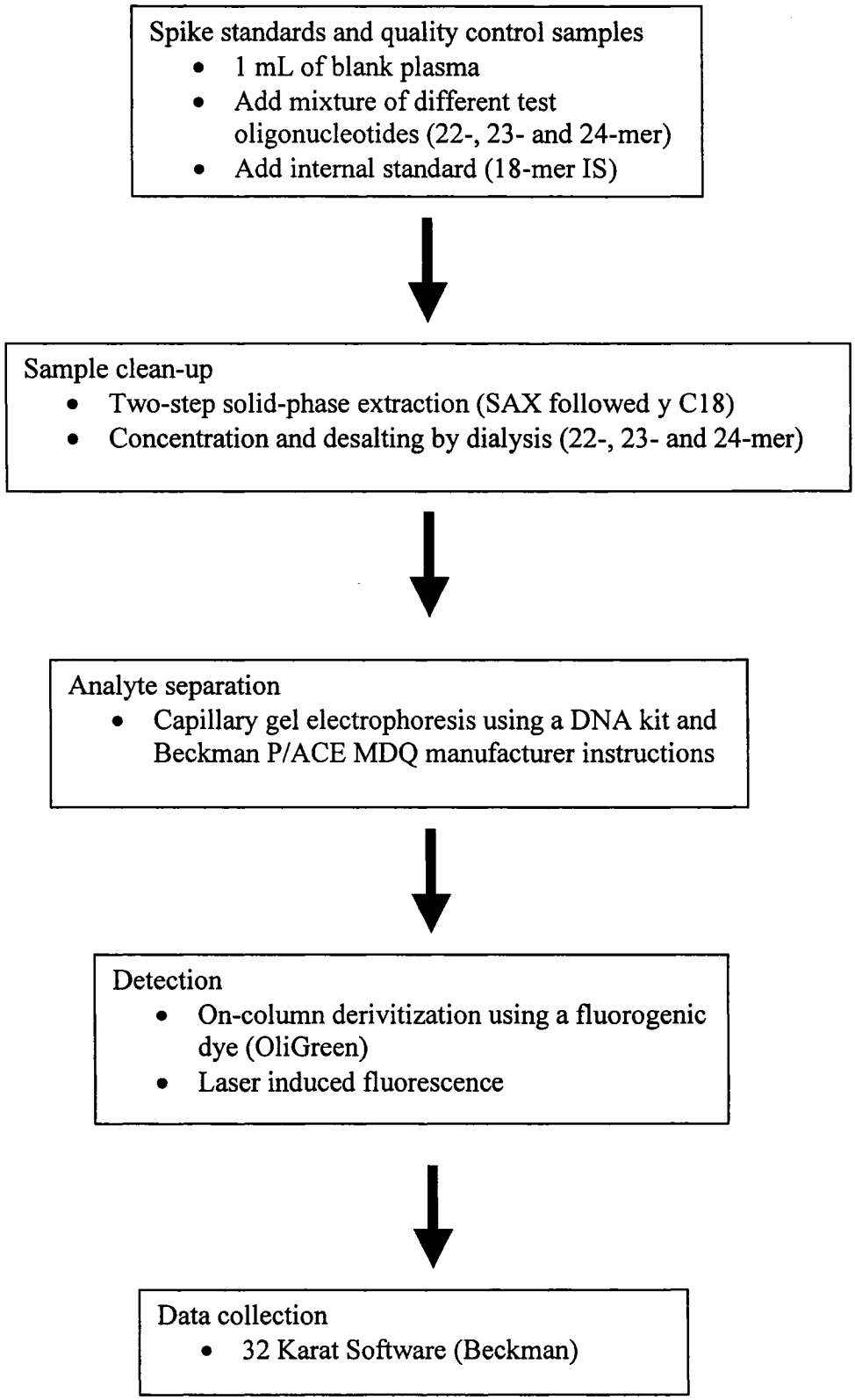
	15 psi applied to both ends of the capillary
LIF detection:	excitation wavelength: 488 nm
	emission wavelength: 520 nm
Run time:	variable (15 to 20 minutes)

3.2.7 Data Collection and Statistical Analysis of Data

Data collection was performed using 32 Karat Software, version 5.0, from Beckman Coulter.

Statistical analyses included weighted (1/concentration) linear fit regression of the normalized areas against the expected concentration for each standard using SigmaPlot (version 2000). The analyte areas were corrected for migration time and area of internal standard.

Table 3-1: Work flow of analytical procedure



3.2.8 System Suitability

The suitability of the CE system was determined by injecting a calibration standard solution, in triplicate, following the first gel fill of each run, then in single at the end of sample analysis; if necessary (*i.e.*, the acceptance criteria were not met with one injection), two additional injections were made.

The parent compound, the metabolites and the internal standard were to be adequately resolved from one another so as to allow accurate quantitation. The analyte was to maintain a comparatively constant relative migration time when compared to the internal standard migration time by visually assessing the electropherograms. The coefficient of variation (CV) of the average normalized area of analyte for the triplicate injections was to be $\leq 5\%$, with a difference between the first and last set of injections to be within $\pm 5\%$ if the calibration standard solution was only injected once at the end of sample analysis, or within $\pm 10\%$ if the calibration standard solution was injected in triplicate.

The 24-mer parent analyte, the n-1 and n-2 metabolites and internal standard were adequately resolved from one another. The parent and the metabolites maintained a consistent relative migration time when compared to the internal standard migration time; this was qualitatively assessed by visual assessment of the electropherograms.

The CV for the average normalized area of the 24-mer parent analyte over the triplicate injections was $< 5\%$, with a difference between the first and last set of injections within $\pm 10\%$.

Acceptance criteria with respect to system suitability were met. Results are presented in Table 3-2.

Table 3-2: System Suitability using a sample of 25.0 ng/mL with three repeated measurements. There were 40 separations between the set of replicates.

Replicate		Migration time of 24-mer (minutes)	24-mer peak area	Migration time of 18-mer (minutes)	18-mer peak area	Nomalized area
First three replicates						
1	CAL 1	8.108	1127424	7.408	943656	1.092
2	CAL 2	8.108	626913	7.412	493436	1.161
3	CAL 3	7.646	528053	6.992	428036	1.128
					Mean NA:	1.1271
					SD:	0.03494
					N:	3
					%CV:	3.1
Last three replicates						
4	CAL 4	7.383	336077	6.742	271972	1.128
5	CAL 5	7.421	398712	6.775	327316	1.112
6	CAL 6	7.571	288602	6.912	236294	1.115
					Mean NA:	1.1185
					SD:	0.00210
					N:	3
					%CV:	0.2
					% Difference:	-0.8

3.3 Validation and Results and Discussion

3.3.1 Selectivity

Selectivity was assessed by analyzing six different lots of double blank plasma. In addition, plasma with the internal standard and parent or metabolites (internal standard single blank), and with parent and metabolites, and no internal standard (analyte single blank) were measured in duplicate.

Any response at the migration time of the test article, for at least five of the six lots of the double blank samples, was to be $\leq$ 25% of the response of the lowest standard in the curve. Any response at the internal standard migration time was to be $\leq$ 5% of the mean response of the internal standard obtained from all the standards accepted in the calibration curve.

For the internal standard single blank, any response at the analyte migration time was to be $\leq$ 25% of the response of the lowest standard in the curve.

For the analyte single blank, any response at the internal standard migration time was to be $\leq$ 5% of the mean response of the internal standard obtained from all the standards accepted in the calibration curve.

There was no interference in the double blanks or in the single blanks at the migration times of the 24-mer parent analyte, the n-1 and n-2 metabolites or internal standard.

Representative electropherograms are presented in Figure 3-1.

Acceptance criteria with respect to selectivity were met.

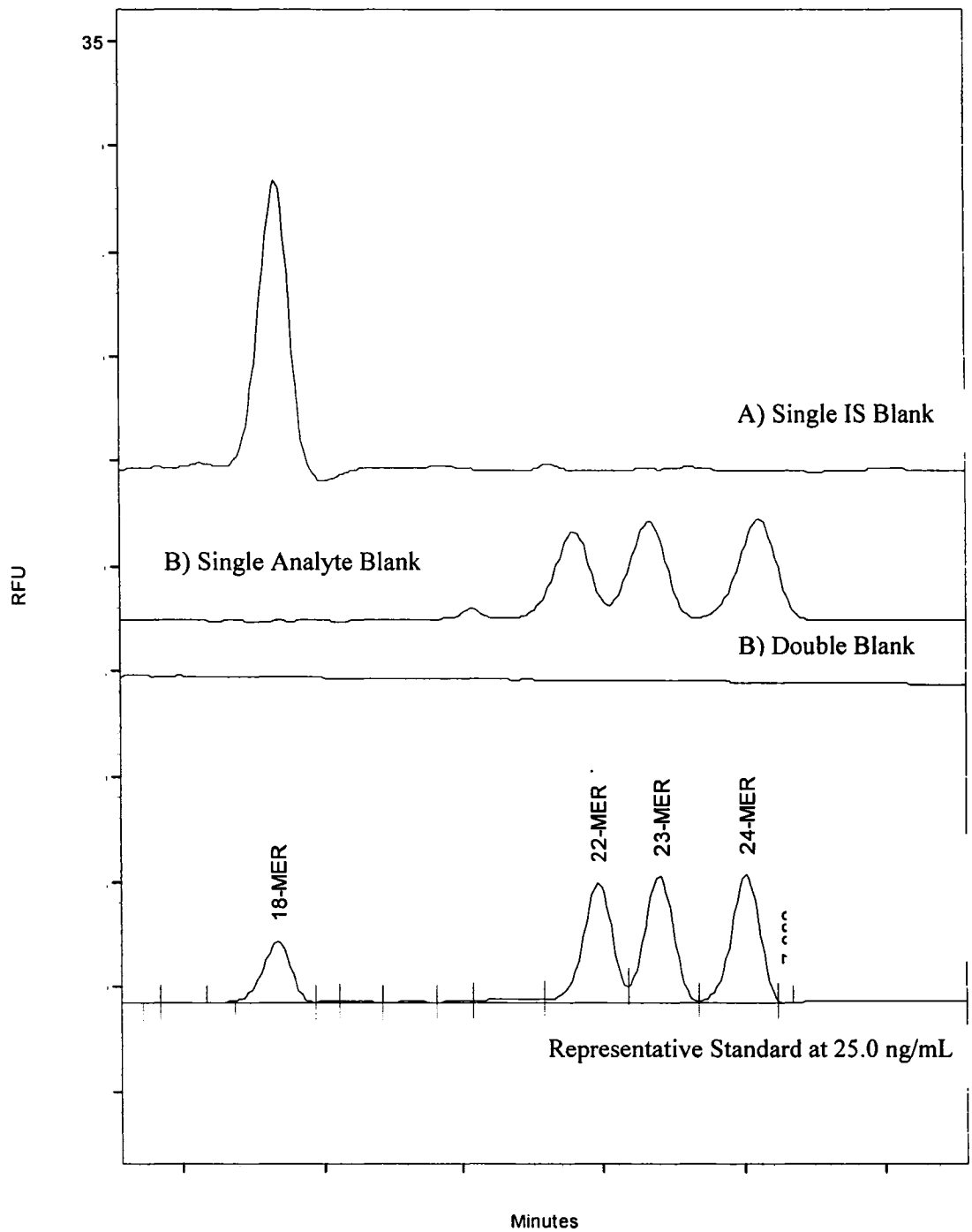


Figure 3-1: Representative blank electropherograms (traces have been moved on both X- and Y-axis). A) Electropherogram of a single analyte blank. Single analyte blank was spiked with no IS and only analyte. For all injections, OliGreen™ was diluted 500 fold. B) Electropherogram of a single IS blank. Single IS blank was spiked with IS and no analyte. C) Electropherogram of a double blank sample. Double blank samples were spiked with water instead of IS and no analyte.

3.3.2 Carry-over

Carry-over was assessed by injecting a processed blank plasma sample immediately after the injection of the highest concentration standard in the calibration curve.

Any response at the migration time of the analyte was to be $\leq$ 25% of the response of the LLOQ standard and any response at the migration time of the internal standard was to be $\leq$ 5% of the mean response of the internal standard obtained from all the standards accepted in the calibration curve.

There were no peaks at the migration times of the 24-mer parent analyte, the n-1 and n-2 metabolites or the internal standard. Data not shown but similar to Figure 3-1.

Acceptance criteria for with respect to carry-over were met.

3.3.3 Linearity

In the first occasion, linearity was assessed for the parent 24-mer compound and its n-1 and n-2 metabolites with nine non zero standards covering the nominal 24-mer and n-1 and n-2 metabolite concentration range of 0.250 to 25.0 ng/mL.

For the parent 24-mer analyte, linearity was considered acceptable if the coefficient of determination (r^2) was $\geq$ 0.98 and the back-calculated concentrations for at least six standards was within ±20% of their expected values (±25% for the LLOQ standard). Standards, which had back-calculated values outside this range, were to be removed from the calibration data. In the event that the lowest and/or highest calibration standard was removed, the range was to be defined by the next highest/lowest calibration standard.

The coefficients of determination (R^2) for the 24-mer parent and its n-1 and n-2 metabolites on the first occasion were 0.997, 0.998 and 0.999, respectively, and

individual back calculated concentrations for the parent and metabolite standards were within ±20% of their expected values, except for the LLOQ standards, which were within ±25% of their expected values. Acceptance criteria for the 24-mer parent with respect to linearity were met.

From Table 3-3, we see that not using a weighted (1/concentration) linear fit regression, makes the relative error data heteroscedastic (with the highest errors at the lower end of the curve). When a weighting factor is applied, the relative errors are more consistent (homoscedastic data). The weighting factor makes the concentrations at the lower end of the curve more usable them more

Representative electropherograms are presented in Figure 3-3.

Calibration curves for the 24-mer parent and 23-mer (n-1) and 22-mer (n-2) metabolites are presented in Figure 3-2. Results are presented in Table 3-3.

Table 3-3: Linear response for the 24-mer parent compound

Expected concentration (ng/mL)	**Calculated concentration (ng/mL)**		**Relative error (%)**	
	Weighted 1/concentration	**Un-weighted 1/concentration**	**Weighted 1/concentration**	**Un-weighted 1/concentration**
0.250	0.229	0.0275	-8.4	89.0
0.500	0.514	0.321	2.7	35.8
0.750	0.718	0.532	-4.2	29.0
1.00	0.943	0.765	-5.7	23.5
2.50	2.74	2.62	9.7	-4.8
5.00	5.23	5.18	4.5	-3.7
10.0	10.2	10.3	1.5	-2.6
15.0	15.8	16.0	5.1	-7.0
25.0	23.7	24.2	-5.2	3.1
Correlation of determination (R^2)				0.997

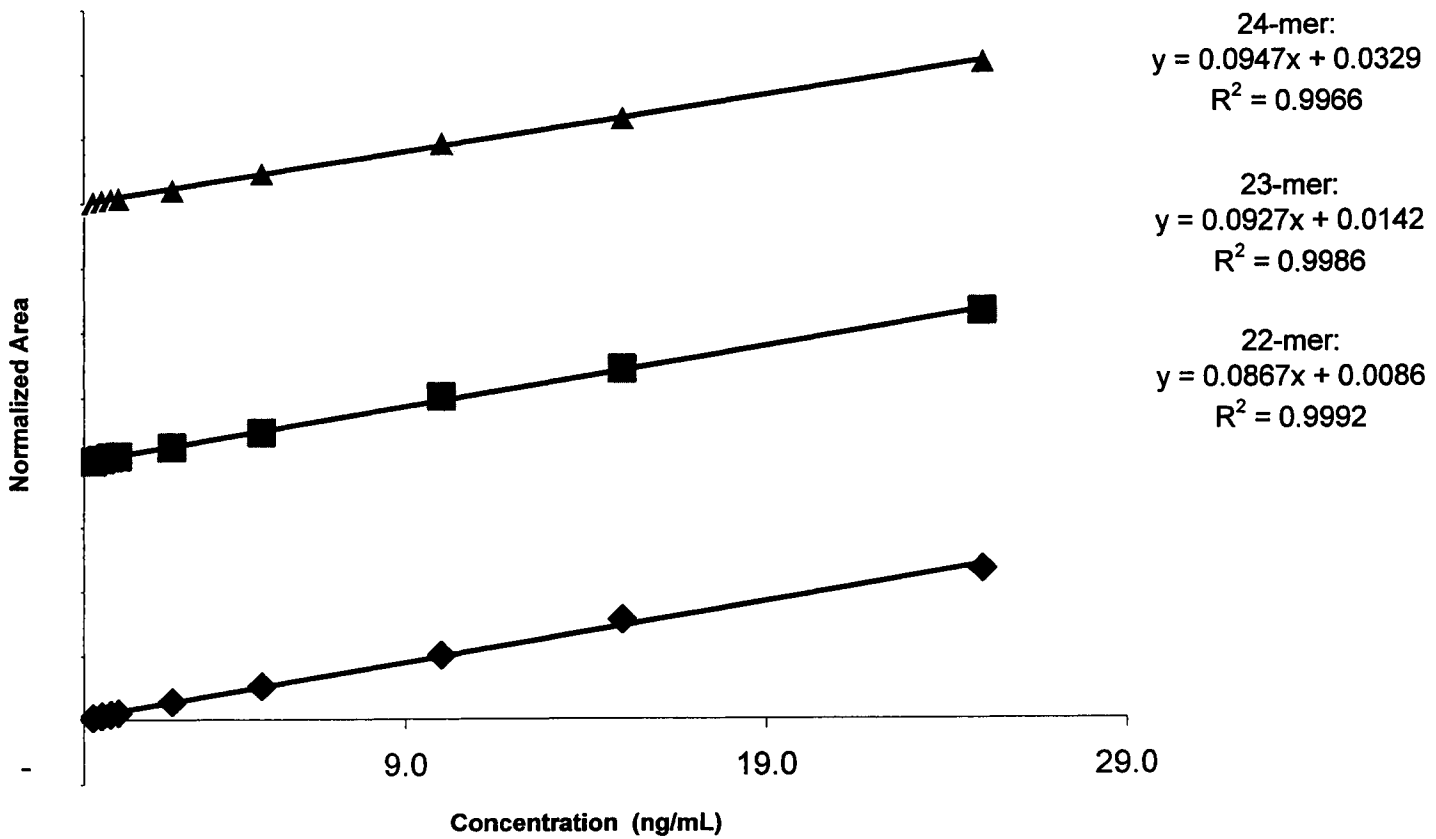


Figure 3-2: Calibration curve for the 24-, 23- and 22-mer oligonucleotide in plasma by CGE-LIF (expected concentration range of 0.250 to 25.0 ng/mL) (the individual calibration curves have been off-set for clarity).

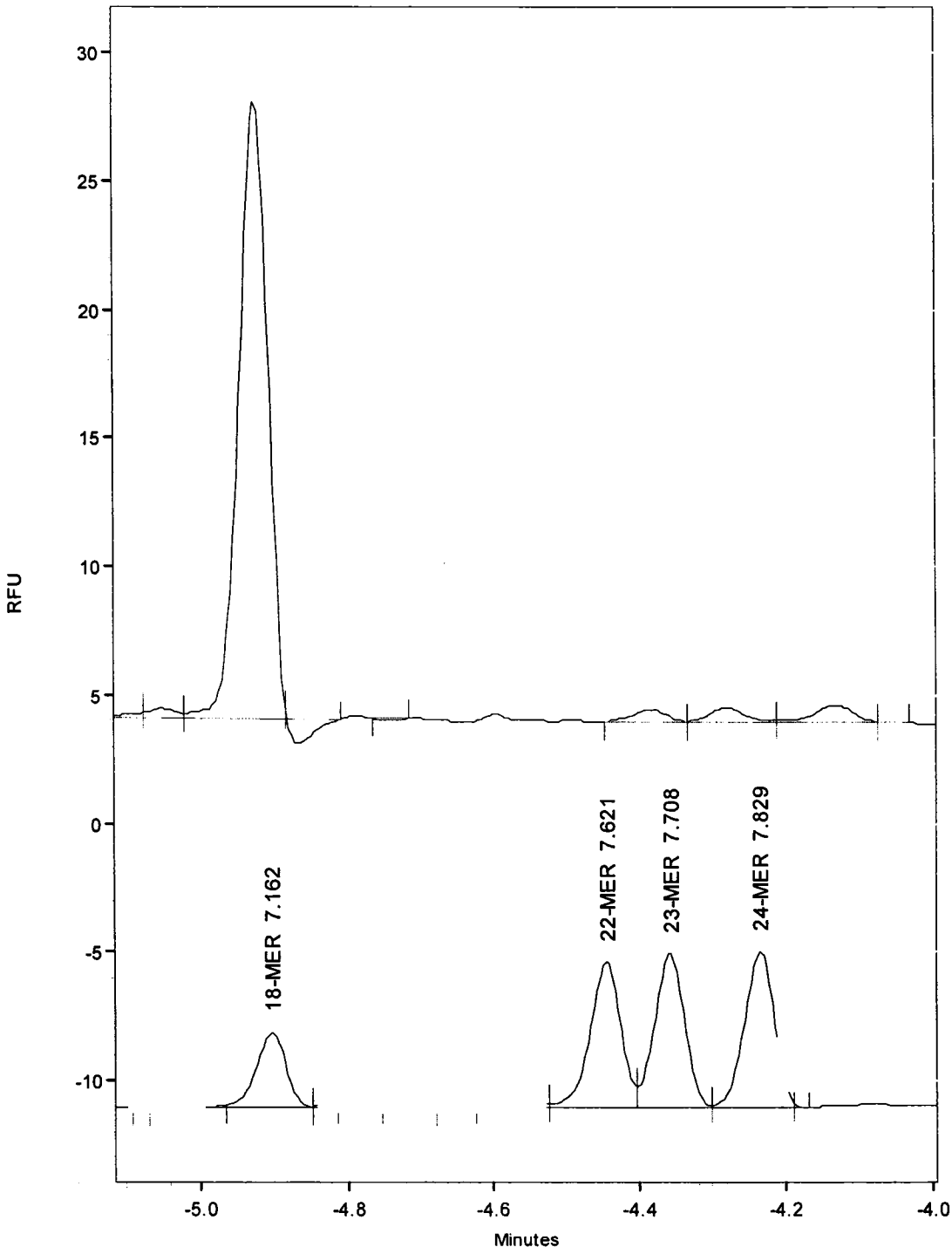


Figure 3-3: Representative electropherogram of 22-, 23- and 24-mer standards (expected concentration 0.250 ng/mL and 25.0 ng/mL respectively, IS concentration-10 ng/mL)

3.3.4 Intra-assay Precision and Accuracy

Quality control samples were spiked at six different concentrations (1.00, 1.50, 2.00, 5.00, 15.0 and 20.0 ng/mL) with the 24-mer parent analyte, n-1 and n-2 metabolites. Six samples were analyzed for each of the concentrations.

For accuracy, the average back-calculated concentration of at least five of the six QC samples for the 24-mer parent, at each level, was to be within ±20% of their expected values. For precision, the CV for at least five of the six QC samples for the 24-mer parent, at each level was to be ≤ 20%.

The mean relative errors for the 24-mer parent for QC samples were -8.1, 10.7, -11.4, -3.5, 9.9 and 5.3%, respectively, with CVs of 9.1, 6.9, 7.1, 8.4, 7.4 and 12.5%, respectively. Acceptance criteria with respect to intra-assay precision and accuracy for the 24-mer parent were met. There are no acceptance criteria for the metabolites.

Results are presented in Table 3-4.

Table 3-4: Intra-assay precision and accuracy

Expected concentration (ng/mL)	Average relative error (%)	CV (%)
1.00	-8.1	9.1
1.50	10.7	6.9
2.00	-11.4	7.1
5.00	-3.5	8.4
15.0	9.9	7.4
20.0	5.3	12.5

3.3.5 Inter-assay Precision and Accuracy

On three separate occasions, QC samples were spiked at three different concentrations (5.00, 15.0 and 20.0 ng/mL) with the 24-mer parent analyte and the n-1 and n-2 metabolites. Six low (nominal 24-mer and metabolite concentration 5.00 ng/mL), six middle (nominal 24-mer and metabolite concentration 15.0 ng/mL) and six high (nominal 24-mer and metabolite concentration 20.0 ng/mL) QC samples were analyzed.

On each occasion, each set of QC samples was to meet the acceptance criteria cited in the intra-assay precision and accuracy section. The global 24-mer analyte average relative error of the three batches was to be within ±20% and the global 24-mer analyte CV was to be $\leq$ 20%, with at least five of the six QC samples from each occasion.

On each of the three occasions, the mean relative errors for all the 24-mer parent QC samples were within acceptance criteria. The global mean 24-mer parent relative errors, for the low, middle and high QC samples, were 0.3, 10.9 and 3.0%, respectively, and the global coefficients of variation were 11.2, 8.3 and 9.1%, respectively.

Acceptance criteria with respect to inter-assay precision and accuracy for the 24-mer parent were met. There are no acceptance criteria for the metabolites. Results are reported for information purposes only.

Results are presented in Table 3-5.

Table 3-5: Inter-assay precision and accuracy (n = 6 spread across 3 occasions)

Expected concentration (ng/mL)	Global relative error (%)	Global CV (%)
5.00	0.3	11.2
15.0	10.9	8.3
20.0	3.0	9.1

3.4 Conclusions

In conclusion, the results of this study demonstrated the feasibility of validating an assay for the measurement of oligonucleotides in plasma by CGE with LIF detection. Linearity, selectivity, carry-over, intra- and inter-assay precision and accuracy pre-validation met acceptance criteria. A lower limit of quantitation of 250 pg/mL was achieved in plasma, which is approximately a 100-fold sensitivity enhancement in comparison to existing CGE-UV methods (70 ng/mL).[12]

This method meets the dire need to analyze oligonucleotides with their metabolites in plasma in order to advance pre-clinical and clinical studies with therapeutic oligonucleotides.

Chapter 4

Capillary Gel Electrophoresis with Laser Induced Fluorescence: the Future of Oligonucleotide Quantitation

4.1 Introduction

Considering the popularity of ASO, the FDA has decided to tighten the criteria on approving new therapeutic drugs. They now require the preclinical trials to show the bioavailability of the drug as well as the fate of the drug candidate in the test subject. They require the determination of the drug concentration at the low pg/mL level in many biological matrices (blood, muscle tissue, liver, kidney and spleen). Also, the FDA wants to see the concentrations of the metabolites of these ODNs in the test subjects.

Thus, the development of ASOs into therapeutic agents has prompted the development of bioanalytical methods for their quantitation in various biological fluids and tissues. These methods are necessary for the characterization of the pharmacokinetic (PK) and pharmacokinetic/pharmacodynamic (PK/PD) behavior of these drugs in support of pre-clinical and clinical development as well as the FDA's demands.

Many different analytical methods are available for the quantitation of ASO, however there are many limitations to these methods.

4.2 Radiolabel tracer methods

Labeling oligonucelotides with radiotracers followed by scintillation counting for detection was one of the first analytical techniques used for determining antisense ODNs in biological matrices in support of PK and PK/PD pre-clinical trials.[35-36] The disadvantages associated with radiotracer label methods are low sensitivity, poor selectivity as the degradation products or metabolites cannot be distinguished from the parent, high costs associated with radioisotopes and inability to detect metabolites. Hence the PK parameters generated from such studies are difficult to interpret. In one study with the ASO G-3139 by Genta using tritium as the radiolabel, the PK data indicated that the short distribution half-life of the drug was 0.03 hours and the longer terminal elimination half-life was 9.8 hours. However, further investigational studies utilizing capillary gel electrophoresis indicated high concentrations of the ODN at time points greater than 2 hours. This is an indication that the parent ASO metabolizes quickly with elimination of the tracer however, the metabolite(s) take longer to eliminate.[54] Other inconveniences are problems related to the handling and disposal of radioactive material and the restriction of this technique to pre-clinical trials.[35-36]

4.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) is one of the most widely used separation methods due to its availability and robustness. Ion-pair reversed-phase chromatography and strong anion-exchange (SAX) chromatography have been used for the analysis of oligonucelotides.[35,43-44] Both are good techniques for the separation of nucleic acids since they are charged as a result of the backbone phosphate group. The

regular spacing of the phosphate group along the oligonucleotide backbone makes the separation possible based on net negative charge. Unfortunately, poor resolution has been reported with these techniques. Both ultraviolet (UV) and fluorescence detection can be coupled to an HPLC, the sensitivity with fluorescence being greater than that of UV. However, use of either UV or fluorescence detection can only bring the detection limit down to high nanogram levels.[35] Dionex Corporation has introduced a new line of DNAPac® columns (that include guard columns, analytical columns, etc) that are claimed to provide up to single base separation.[41] The lower limit of detection, or LLOQ, of the methods using these columns has yet to be published. As for sample clean-up procedure, a simple liquid-liquid extraction using phenol-chloroform is sufficient when using HPLC type separation.[35] The disadvantage is that this method can not meet the increasing demand of the FDA to go to pg/mL levels, also the metabolites separation from the parent is not well resolved beyond n-1 at the most.

4.4 Liquid chromatography tandem mass spectrometry (LC/MS)

There is currently an explosion in the ever expanding field of liquid chromatography tandem mass spectrometry (LC/MS) for solving many current analytical problems. LC/MS is a more attractive alternative to traditional analytical separation techniques considering it offers such features as enhanced sensitivity and performance, speed, high throughput and reliability. The use of LC/MS for the analysis of ODNs has not been very successful due to the multiple charge states that oligonucelotides form in an electro-spray. This has prohibited its use for sensitive quantitation analysis. An additional

disadvantage of LC/MS is the high price of the instrumentation. On the other hand, LC/MS has been shown to be a useful tool for metabolite characterization.[35]

4.5 Hybridization-based enzyme-linked immunosorbent assays

Hybridization assays provide great sensitivity with reported lower limits of quantitation on the order of low pg/mL with high sample throughput using minimal sample clean-up.[42] Reagent and instrument costs for this type of analysis are very low, considering the most expensive piece of equipment is a UV or fluorescence plate reader. The critical disadvantage of hybridization based assays is their inability to distinguish a parent oligonucelotide from its metabolites. This may result in cross-reactivity of the metabolites during the quantitation of the parent compound and result in an over-estimation of the parent, a point the FDA is encouraging biotechnology and pharmaceutical companies avoid. Still, these highly sensitive hybridization assays can be used to characterize the elimination phase of the ODNs from the plasma for the assessment of exposure-response correlations or PK/PD in animal trials.[35] Ultimately, this will enable researchers to assign more realistic doses for their clinical trials.

4.6 Capillary gel electrophoresis with ultraviolet detection

Capillary gel electrophoresis (CGE) is similar to HPLC in its instrumentation and operation, but differs in the principle of separation, CE has high resolving power, and low mass detection limits [55] when coupled with either ultraviolet (UV) or fluorescence detection. Separation of intact ODNs as well as related metabolites that differ by only one or two base units are readily accomplished and is CGE's primary advantage.

Previously validated methods for the quantitation of therapeutic ss-ODNs using CGE-UV at 260 nm have demonstrated lower limits of quantitation of approximately 70 ng/mL in plasma and urine.[35,56] CGE-UV is not as sensitive as other analytical techniques such as hybridization assays. Since fluorescence is a more sensitive detection mode than UV, coupling CGE to laser induced florescence (LIF) detection could potentially solve the sensitivity issues related to CGE-UV while also alleviating some of the sample preparation constraints.

4.7 CGE with LIF detection, the future

As previously discussed, CGE is a powerful and efficient analytical separation technique and it continues to be popular in biomedical applications.[57-60] Reasons for its popularity include nanoliter injection volumes and rapid analysis times. Coupling CGE with laser-induced fluorescence (LIF) can allow extremely sensitive online detection of tagged or naturally fluorescent compounds in the attomolar range. This has led to a few biological applications reported using CE-LIF to quantitate fluorescently labeled oligonucleotides.[61-62] OliGreen™, an ultrasensitive fluorescent nucleic acid stain, was recently introduced by Molecular Probes as a novel reagent for the quantitation of ss-DNA fragments and small-molecular-mass ODN's. Sensitivities in excess of 10 000-fold compared with UV absorbance have been achieved with the standard spectrofluorometer.[52] The successful separation of single-stranded ODNs using the fluorescent dye Oligreen™ was first reported by Reyderman and Stavchanscky in 1996.[51] The results of their experiments show limits of detection of 1 ng/mL with the LIF detection, compared with 70 ng/mL with the UV detection.[56-59]

The method we have developed and described within this thesis has reported lower limits of detection in the order of 250 pg/mL, an order of magnitude more sensitive than that reported by Reyderman and Stavchanscky in 1996.[51] Therefore it is clear that this novel approach to oligonucleotide quantitation has great potential to dominate the analytical methods for determination and accurate quantitation of ODNs in biological matrices to meet the increasing demands of the FDA.

4.8 Conclusion

DNA-based biopharmaceuticals have emerged as a new class of therapeutic drugs that target disease progression at different levels of gene expression using compounds of similar nature so that disease associated proteins can be prevented from forming. Different types of therapeutic oligonucleotides exist and include plasmids containing transgenes, single stranded antisense oligonucleotides, ribozymes, DNAzymes, aptamers and small interfering RNAs (siRNAs). To accurately characterize the PK and PK/PD behavior of these drugs, bioanalytical methods have been developed for their quantitation such as radiotracer methods, HPLC, CGE, HPLC-mass spectrometry, and hybridization-based enzyme-linked immunosorbent assays. In order to satisfy the stringent demands of the regulatory agencies and accelerate the approval for these new therapeutic drugs, the future of oligonucleotide quantitation lies in CGE-LIF, more specifically, the CGE-LIF method described within this thesis.

Chapter 5

Conclusions and Future Directions

DNA-based biopharmaceuticals have emerged as a new class of therapeutic drugs that target disease progression at the genomic level. The development of ASOs into therapeutic agents has prompted the development of bioanalytical methods for their quantitation in various biological fluids and tissues for the characterization of the pharmacokinetic and pharmacokinetic/pharmacodynamic behaviour of these drugs in support of pre-clinical and clinical development. Currently, a few bioanalytical methods are available for the quantitation of ASOs in biological matrices (radiotracer methods, HPLC, CGE, HPLC-mass spectrometry, and hybridization-based enzyme-linked immunosorbent assays). The ideal method should be able not only to quantify the parent oligonucleotide, but its chain-shortened metabolites (n-1, n-2, etc) as well, with great sensitivity. CGE offers the greatest selectivity from the above-mentioned methods and coupled to an LIF detection system, has the potential to offer great sensitivity as well.

For these reasons, the feasibility of developing a method for determining short single-stranded oligonucleotide using capillary gel electrophoresis with laser induced fluorescence was investigated. Results from the method development and optimization experiments indicate that the ideal nucleic acid stain to use for quantifying these oligonucleotides is OliGreen^{TM} with a 500-fold dilution. The CE conditions for analyzing short single-stranded oligonucleotides with CGE-LIF are ideal with a capillary temperature of 60°C. The gel needs to be saturated with the dye prior to sample injection.

Subsequently, the method was validated in human plasma following "Guidance for the Industry, Bioanalytical Method Validation" issued by the "United States Department of Health and Human Services, FDA Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM)" issued in "May 2001". The method was validated for a 24-mer parent oligonucleotide in human plasma (EDTA) with respect to selectivity, carry-over, linearity, lower limit of quantitation (LLOQ), and intra- and inter-assay precision and accuracy. The data generated for the 23-mer and 22-mer metabolites were for qualitative purposes only since there are no acceptance criteria for metabolites. A lower limit of quantitation of 250 pg/mL was achieved in human plasma, which is approximately a 100-fold sensitivity enhancement in comparison to existing CGE-UV methods (70 ng/mL).[12]

In conclusion, the results of this study demonstrated the feasibility of validating an assay for the measurement of oligonucleotides in human plasma by CGE with to LIF detection.

This method meets the dire need to analyze oligonucleotides with their metabolites in plasma in order to advance pre-clinical and clinical studies with therapeutic oligonucleotides.

Blood, and plasma, are not the target for antisense oligonucleotides but carry the therapeutic to the target organs. We can also find them in various tissues such as kidney, liver, feces and urine. For future reference, it would be necessary to also develop a method for quantifying oligonucleotides in these tissues. Also, to acquire a more accurate understanding of the dye-oligonucleotide interaction, it would be useful to investigate the structure of the fluorogenic dye used, OliGreen™, as well to conduct

experiments to determine the chemistry between the dye and the short single-stranded oligonucleotide.

Also, I would propose, developing a method combining the sensitivity of a hybridization assay and the selectivity of CGE-LIF. The procedure would involve the utilization of a biotinylated template probe (complementary to the investigated oligonucleotide) and streptavidin-coupled Dynabeads®. An un-extracted plasma sample containing the analyte incubated with biotinylated template probe and streptavidin-coupled Dynabeads® would result in hybridization with the template probe and capture by the beads. Using a magnet to retain the Dynabeads® would allow plasma proteins and lipids to be removed while the hybridized oligonucleotide is left behind. The hybridized oligonucleotide can be freed by a simple heat denaturation step and directly injected onto a CGE-LIF system for analysis. The combination of hybridization and CGE will significantly reduce the total analysis time of oligonucleotides in plasma since no extraction would be required.

References

[1] Stryer, L., Biochemistry, Fourth Edition, W.H. Freeman and Company, United States, (1997).

[2] Crooke S. Current Molecular Medicine, 2004, 4, 465-487.

[3] Crooke S. Annual Review of Medicine, 2004, 55, 61-95.

[4] Patil SD, Rhodes DG, Burgess DJ. AAPS Journal. 2005; 07(1), Article 9.

[5] Crooke S, Antisense Nucleic Acid Drug Development, 1998, 8, 115-122.

[6] Stull RA, Szoka FC Jr, Pharm. Res., 1995, 12, 465-483.

[7] Tombelli S, Minunni M, Mascini M, Biosensors and Bioelectronics, 2005, 20, 2424-2434.

[8] Leeds, J et al. Analytical Biochemistry, 1996, 235(1), 36-43.

[9] Watson J D; Crick F H, Nature (1953), 171(4361), 964-967.

[10] http://nobelprize.org.

[11] Avery O T, MacLeod C M and McCarty M, Journal Exp. Med., (1944), 79, pp. 137–158.

[12] Dixon B, Current Biology (2003), 13(8), R295-R296.

[13] Stein, C.A., Subasinghe, C., Shinozuka, K., Cohen, J.S., Nucleic Acids Res., 1988, 16, 3209-3221.

[14] Zamecnik, P.C; Stephenson, M.L., Proceedings of the National Academy of Sciences of the United States of America, 1978, 75(1), 280-284.

[15] Kurreck, J, European Journal of Biochemistry, 2003, 270(8), 1628-1644.

[16] Hughes, M.D., Hussain, M., Nawaz, Q., Sayyed, P., Akhtar, S., Drug Discov Today. 2001;6, 303-315.

[17] Jansen B; Zangemeister-Wittke U. Lancet Oncology (2002), 3(11), 672-683.

[18] Uhlmann E; Peyman A. Chemical Reviews (Washington, DC, United States) (1990), 90(4), 543-84.

[19] Lysik, M.A., Wu-Pong, S., Journal of Pharmaceutical Sciences, 2003, 92(8), 1559-1573.

[20] Patil, S.D., Rhodes, D.G., Burgess, D.J., AAPS Journal, 2005, 07(1), Article 9.

[21] Le Doan, T., S.T.P. Pharma Sciences, 2001, 11, 75-82.

[22] Shoji, Y., Akhtar S., Periasamy, A., Herman, B., Juliano, R.L., Nucleic Acids Res., 1991, 19, 5543-5550.

[23] Jansen, B., Zangemeister-Wittke, U., The Lancet Oncology, 2002, 3(11), Issue 2, 672-683.

[24] Arnedo, A., Campanero, M.A., Espuelas, S., Renedo, M.J., Irache, J.M., Journal of Chromatography, A, 2000, 871(1+2), 311-320.

[25] Chirila, T.V., Rakoczy, P.E., Garrett, K.L., Lou, X., Constable, I.J., Biomaterials, 2001, 23(2), 321-342.

[26] Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., Baker, B.F., Journal of Biological Chemistry, 2003, 278(9), 7108-7118.

[27] Wu, H., Lima, W.F., Zhang, H., Fan, A., Sun, H.; Crooke, S.T., Journal of Biological Chemistry, 2004, 279(17), 17181-17189.

[28] Crooke, S.T., Antisense Nucleic Acid Drug Dev., 1999, 9, 377-379.

[29] Stein, C.A., Subasinghe, C., Shinozuka, K., Cohen, J.S., Nucleic Acids Res., 1988, 16, 3209-3221.

[30] Campbell, J.M., Bacon, T.A., Wickstrom, E., Journal of Biochemical and Biophysical Methods, 1990, 20(3), 259-267.

[31] A Levin, A.A., Biochimica et Biophysica Acta, Gene Structure and Expression, 1999, 1489(1), 69-84.

[32] Aboul-Fadl, T., Current Medicinal Chemistry, 2005, 12(19), 2193-2214.

[33] Azad, R.F., Driver, V.B., Tanaka, K., Crooke, R.M., Anderson, K.P., Antimicrobial agents and chemotherapy, 1993, 37(9), 1945-1954.

[34] Azad R.F., Brown-Driver V., Buckhe,t R.W. Jr., Anderson K.P., Antiviral research, 1995, 28(2), 101-111.

[35] Yu, R Z., Geary, R S., Levin, A.A., Current Opinion in Drug Discovery & Development, 2004, 7(2), 195-203.

[36] Agrawal, S., Temsamani, J., Galbraith, W., Tang, J., Clinical Pharmacokinetics, 1995, 28(1), 7-16.

[37] Sands, H., Gorey-Feret, L.J., Cocuzza, A.J., Hobbs, F.W., Chidester, et al., Molecular Pharmacology, 1994, 45(5), 932-943.

[38] Cossum, P.A., Truong, L, Owens, S.R., Markham, P.M., Shea, J.P., et al., Journal of Pharmacology and Experimental Therapeutics, 1994, 269(1), 89-94.

[39] Stuart, D.D., Kao, G.Y., Allen, T.M., Cancer Gene Therapy, 2000, 7(3), 466-475.

[40] Chen, S.H., Gallo, J.M., Electrophoresis, 1998, 19(16-17), 2861-2869.

[41] Dionex Corporation, 1992-1998, 2001, Document number 034411, revision 7, 2001.

[42]. Yu, R.Z., Baker, B, Chappell, A., Geary, R.S., Cheung, E., et al., Analytical Biochemistry, 2002, 304(1), 19-25.

[43] Dovichi N. et al., Angewandte Chemie, International Edition, 2000, 39(24), 4463-4468.

[44] Geary, R.S., Yu, R.Z., Watanabe, T., Henry, S.P., Hardee, G.E., et al., Drug Metabolism and Disposition, 2003, 31(11), 1419-1428.

[45] McKeon, J., Khaledi, M.G., Electrophoresis, 2001, 22(17), 3765-3770.

[46] Altria, K.D, Journal of Chromatography, A, 1999, 856(1 + 2), 443-463.

[47] Hjerten, S., Chromatographic reviews, 1967, 9 (2), 122-219.

[48] Mikkers F., Ringoir S., De Smet R., Journal of chromatography, 1979, 162(3), 341-350.

[49] Jorgenson J.W., Lukacs K.D., Clinical chemistry, 1981, 27(9), 1551-1553.

[50] Baker, D.R., Capillary Electrophoresis, A Wiley-Interscience Publication, John Wiley and Sons, Inc., New York 1995.

[51] Reyderman, L., Stavchansky, S., Journal of chromatography A, 1996, 755, 271-280.

[52] Molecular probes on-line catalog, Molecular Probes, Inc., Copyright 2005

[53] Zipper, H et al, Nucleic Acids Res., 2004, 32(12):e103.

[54] Yu RZ, Geary RS, Leeds JM, Watanabe T et al. (2001) Journal of Pharmacological Sciences 90(2):182-193

[55] Cheng Y-F, Dovichi N (1988) Science 242:562-564

[56] Geary RS, Yu RZ, Watanabe T, Henry SP, Hardee GE et al. (2003) Drug Metabolism and Disposition 31(11):1419-1428

[57] Hegaard NH, Nilsson S, Guzman NA (1998) Journal of Chromatography B 715:29–54

[58] Liu LM, Tzeng YM (1998) Journal of Food and Drug Analysis 6:391–403

[59] Ulrich O, Grune T (1997) Journal of Chromatography B 697:243–249

[60] Phillips TM, Dickens BF (1998) Electrophoresis 19:2991–2996

[61] Cheng J, Shoffner MA, Mitchelson KR (1996) Journal of Chromatography A 732:151–158

[62] Chen H, Manabe T, Terabe S (1994) Journal of Microcolumn Separation 6:39–543