ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1127–1135

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Qualitative and quantitative measurements of oligonucleotides in gene therapy: Part II in vivo models

H.K. Tewary [a], P.L. Iversen [a,b,*]

[a] *Department of Pharmacology, University of Nebraska Medical Center, 600 South 42nd Street, Omaha NE 68198-6260, USA*
[b] *Eppley Institute of Cancer Research, University of Nebraska Medical Center, 600 South 42nd Street, Omaha NE 68198-6260, USA*

Received 1 February 1996; accepted 25 August 1996

Abstract

Part I of this review has already been presented on methods of processing and purification of crude or raw samples in cell-culture or cell free systems. Part II of the review focuses on the in vivo models of determination of input oligonucleotides in both non-primates and primates. Emphasis has been given to the techniques developed for quantification of oligonucleotides or their metabolites from biological samples including blood, plasma, serum, urine and other tissues. © 1997 Elsevier Science B.V.

Keywords: Oligonucleotides; Gene therapy; Measurement; Biological samples

1. Introduction

In Part I of this review, we have discussed the in vitro models of detection and determination of synthetic oligonucleotide in cell culture or cell free systems. The in vitro models simply pave the way for development of the in vivo models. During the past couple of years, extensive efforts have been made by researchers worldwide to develop in vivo models for using oligonucleotides in gene therapy or antisense therapy. These models include both non-primates and primates prior to Phase I trial of these therapeutic agents. Hence this part of the review covers most of the work conducted in this area.

* Corresponding author. Tel.: + 1 402 5597115/4540 fax: 1 + 402 5597495.

2. In vivo models

The pharmokinetic analysis of oligonucleotide behavior in vivo is a critical point in the development of biologically active oligonucleotides. Determination of the radioactivity of ^{35}S or ^{32}P located at the 3′-end, 5′-end or middle (mid-way) of the oligonucleotide in a sample of blood/plasma, body tissues, or urine, of animals treated with such radiolabeled oligonucleotides is generally done by either directly counting the radioactivity (liquid scintillation counting (LSC)) or after some purification or processing steps to remove some interfering substances followed by LSC, polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). The concentration is determined by di-

PII S0039-9140(97)01942-5

viding the radioactivity measured in a defined volume of tissue sample by the specific activity of the administered oligonucleotide in cpm μg^{-1} of oligonucleotide [1]. Alternately, the oligonucleotide in the processed samples is further labeled at the 5′- or 3′-end by the nuclide, and subjected to PAGE analysis followed by autoradiography and densitometry. The in vivo models are described separately for non-primates and primates.

3. Radiolabeling

3.1. LSC

For the in vivo study of radiolabeled oligonucleotides injected into animals through different routes (such as intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.)), biological samples such as blood, urine and other body organs are obtained after a predetermined period post-injection of radiolabeled oligonucleotides. Body organs/tissues are generally homogenized, solubilized, and sometimes extracted and decolorized before LSC.

Following this approach, Zendequi et al. [2] used γ-^{32}P labeled and relatively G-rich 38-mer oligonucleotide with a phosphopropyl amine group at the 3′-end to prevent degradation by nucleases in female BALB/cAnNhsd mice by injecting 130 mg kg^{-1} in 0.2 ml PBS i.p. or i.v. via the lateral tail vein. At various time points after injection, the mice were anaesthetized and blood was collected via cardiac puncture into a heparinized syringe. The mice were then sacrificed by cervical dislocation, and body tissues were removed, rinsed in ice-cold PBS, frozen immediately in liquid nitrogen and stored at −70°C until use. Blood and tissue samples were solubilized in Soluene, incubated (37°C, 15 h), H_2O_2 was added to decolorize the tissue, and radioactivity was measured using LSC. Skorski et al. [3] used 26-mer bcr-abl antisense phosphorothioate oligonucleotide in the SCID mouse model, Sands et al. [4] a ^{3}H-labeled 20-mer oligonucleotide analogue in mice, Temsamani et al. [5] end capped ^{35}S-labeled oligonucleotides in mice, and Smidt et al. [6] a 16-mer ^{32}P-labeled amphiphatic cholesteryl-oligonucleotide complexed with low-density lipoprotein (LDL) on male Wistar rats. Goodarzi et al. [7] instead extracted the oligonucleotide from frozen and crushed organs using extraction buffer, phenol, and chloroform, lyophilized and reconstituted with water, before LSC, in a study of a ^{32}P-labeled 20-mer phosphorothioate oligonucleotide injected through different routes in mice.

Goodchild et al. [8] used 20-mer phosphodiester oligonucleotide ^{32}P-labeled both at the 5′-end and internally (mid-way) on New Zealand while male rabbits implanted with a polyethylene cannula in the carotid artery for sampling blood (3 ml) at different times (up to 90 min) after injecting 3.5 ml ($\approx$8.75 mg labeled ^{32}P) into a marginal ear vein of the rabbit. Plasma obtained in 1/10th diluted blood in 3.8% sodium citrate buffer was added to an equal volume 10% SDS and then counted using LSC. Corrections were made using blanks, and they found the quenching effect was in the range of 72–74%. A similar approach to the stability of ^{32}P-labeled 20-mer oligonucleotide was studied in rabbit blood [9]. To study the inhibition of replication and expression of HIV in susceptible living human cells, Zamecnik and Agrawal [10] found that the unmodified ^{32}P-labeled oligonucleotides injected (i.v.) were cleared from the blood of rabbits with a half life of about 8 min. These oligonucleotides were degraded rapidly in different organs (i.e. kidney, liver, intestine, spleen, muscle, lung, bladder and brain). These workers also used ^{35}S-labeled oligonucleotides, and obtained similar results. However, phosphorothioate modified oligonucleotides showed a half-life of intact oligonucleotide of 24–48 h.

3.2. PAGE

For the in vivo study of oligonucleotides by PAGE, blood, urine and body organs are obtained at predetermined intervals post-injection. Organs are generally homogenized, and extracted in suitable buffer to remove interfering substances. The oligonucleotide used is generally ra-

diolabeled before injecting into the animals or it is post-labeled using mostly ^{35}S, ^{32}P radioisotope, before analysis by PAGE followed by autoradiography and densitometry. PAGE analysis is mostly followed to study the metabolism of the oligonucleotide in systemic conditions.

Temsamani et al. [11,5] studied the pharmacokinetics and stability of the 3′- and/or 5′-end capped oligonucleotides ^{35}S-labeled oligonucleotides after injecting mice (30 mg kg^{-1}, i.v.). End capping was done with amino-1,2-propanediol). The oligonucleotide was extracted from the homogenized tissues by phenol extraction before PAGE using 20% polyacrylamide–8 M urea gels. Analysis by PAGE of the stability of the oligonucleotide excreted in urine showed that the 3′-end capped oligonucleotide had no degradation against 5′-end capped olionucleotides after 24 h postdosing. Skorski et al. [3] instead used an indirect approach to determine oligonucleotide used in an in vivo study. They showed that 26-mer bcr-abl antisense phosphorothioate oligonucleotide effectively suppressed leukemia growth in SCID mice injected (i.v.) with the oligonucleotide at 1 mg day^{-1} for 9 days. At various time points after the last injection, oligonucleotide was isolated from single-cell-suspensions of each organ, electrophoresed (15% polyacrylamide–7 M urea), and electroblotted on Nytran membranes which were subsequently hybridized with 26-mer ^{32}P-end labeled sense oligonucleotide at 37°C for 18 h. The isolates were washed twice with SSC–0.1% SDS before autoradiography.

Woolf et al. [12] studied the stability, toxicity, and effectiveness of 25-mer unmodified and phosphorothioate oligonucleotides in *Xenopus* oocytes and embryos in order to elucidate their in vivo stability. They used ^{35}S internally labeled oligonucleotides in order to distinguish between complete degradation and phosphatase or minor exonuclease activity. The oligonucleotides were extracted by homogenization in homogenization buffer containing proteinase K, incubated for 30–60 min at 37–60°C, extracted twice by phenol–chloroform followed by ethanol precipitation from 0.3 M sodium acetate, 10 mM $MgCl_2$ with two volumes of ethanol. The extract was analyzed by PAGE, briefly fixed in 10% acetic acid–40% methanol, then treated with Amersham Amplify before autoradiography. It was shown that the unmodified oligonucleotide in both oocytes and embryos was rapidly degraded with a half life of less than 30 min. These results were consistent with others, obtained using end labeled oligonucleotides. The pharmacokinetics of ^{35}S-labeled 20-mer oligonucleotide in a mouse model [13], a ^{32}P-labeled and 3′-end modified oligonucleotide analogue in mice [14], has been studied using a similar approach.

The in vivo oligonucleotide stability of an internally ^{32}P-labeled 38-mer oligonucleotide in mice was studied in two different ways for blood and tissue samples, respectively [2]. For blood, plasma (10 μl) was first incubated alone at 65°C for 10 min, then 5′-end labeled by [γ^{32}P]ATP using T4 polynucleotide kinase, and extracted with a equal volume of phenol–chloroform (1:1). The aqueous layer was removed, dried in a lyophilizer, and soaked in 400 μl of 85% ethanol for 3 h at room temperature to remove salts and unincorporated ^{32}P. After centrifugation (15 000 × *g*, 5 min), the precipitate was dissolved in water, denatured using 50% formamide at 65%, and analyzed on PAGE (12% polyacrylamide gel, 7 M urea, TEB). Using autoradiography they found no selective loss of oligonucleotide fragments of 10 bases, and greater, under these conditions. For tissue, 50 mg of each sample was homogenized in buffer, extracted with phenol–chloroform (1:1), treated with RNase A, and incubated at 60°C for 1 h with proteinase K. The sample was again extracted with phenol–chloroform, dried and extracted with 85% ethanol, and the isolated precipitate was dissolved in water and 5′-end labeled with [γ^{32}P]ATP using T4 polynucleotide kinase. The PAGE analysis and autoradiography were carried out as for the blood/plasma sample. Oligonucleotide in urine was determined after collecting the urine of animals at different time points after i.v. or i.p. injections, and later analyzed by LSC or by 5′-end labeling followed by PAGE and autoradiography.

A combination of both LSC and PAGE analysis was followed by Inagaki et al. [15] in the kinetic study of TGF-β (24-mer) and c-myc (15-mer) phosphorothioates 5′-end labeled with [γ^{32}P]ATP in rats. At various time points post i.v.

injection of oligonucleotides, animals were sacrificed and blood and organs were removed. The organs were homogenized prior to a determination of total radioactivity. The plasma levels of ^{32}P labeled oligonucleotides in rats decreased by 50% in 5 min post injection, and the oligonucleotide accumulated mainly in the liver. To detect the injected ^{32}P labeled oligonucleotides in the liver, the homogenized liver was treated with proteinase K (2 mg ml^{-1}) in extraction buffer. The samples were then extracted twice with phenol–chloroform and once with chloroform. After ethanol precipitation, the samples were analyzed by electrophoresis on a 20% polyacrylamide–7 M urea gel.

3.3. HPLC and other methods

HPLC of biological samples is generally adopted in order to detect oligonucleotide both qualitatively and quantitatively. Many investigators have used reversed-phase (RP), size exclusion (SE), or strong anion exchange (SAX) methods for both crude biological samples (mostly urine) or after following some purification steps. The retention time of a standard oligonucleotide peak is used to confirm the identity of oligonucleotide in biological samples. However, sometimes HPLC has been used in combination with LSC or PAGE analysis. The HPLC fractions are either counted directly using LSC, or further 5′-end or 3′-end labeled with ^{35}S or ^{32}P radioactive isotope and PAGE is used to confirm the presence of oligonucleotide in such samples. The latter approach (HPLC and PAGE) obviously appears more logical, since oligonucleotide, after passing through systemic conditions, may bind with other constituents present in biological tissues and its retention time may vary. Bigelow et al. [16] reported pharmacokinetic data on antisense oligonucleotide treated mice, based on a method to measure the concentration of oligonucleotide in biological fluids (plasma and urine) employing the criteria of (a) spiking the oligonucleotide to the biological sample (plasma and urine), and (b) retention time of eluted peaks. They described a sensitive (20 nM) and fast (20 min) HPLC method for analysis of a 28-mer antisense oligonucleotide to a region of rev of (HIV-1). Biological samples were prepared by treating with lysis buffer and proteinase K followed by phenol–chloroform–H_2O extraction before HPLC analysis (tetrabutylammonium dihydrogenphosphate (TBAP) was used as the ion-pairing agent). Oligonucleotide recovery of $\approx 80\%$ was reported. Wickstrom et al. [17] used a similar approach of deproteinizing the blood samples drawn from mice treated with 15-mer methylphophonate oligonucleotide antisense to c-myc mRNA before analyzing with reversed-phase liquid chromatography.

The combination of HPLC and LSC has been followed by many investigators. Sands et al. [4] used ^{3}H-labeled 20-mer oligonucleotide analogues in mice to study their comparative biodistribution and metabolism. Mice injected at a dose of 6 mg kg^{-1} (about 6 μCi) were sacrificed at various times after injection. Approximately 200 mg of tissue (kidney, liver and spleen) were treated with GIT to terminate metabolism and denature proteins, sonicated, and diluted with 5 volumes of water followed by extraction with an equal volume of phenol–chloroform (1:1, v/v), and centrifuging at $10\,000 \times g$ for 30 min. this process was repeated a second time to fully extract the organic layer. The aqueous layers from the two extractions were pooled, lyophilized, and resuspended in water for analysis by HPLC. More than 70% of the radioactive nucleic acids in the tissues were recovered and detected by HPLC. For the HPLC analysis, the paired-ion chromatography was performed using a Microsorb MV-C4 column. The eluate from the column was mixed on-line with 4 ml of Ready Flow scintillation cocktail (Beckman) and analyzed using a Betram on-line β-radiation detector. 24 h Urine was collected in metabolic cages, centrifuged through 0.2 μm filters, diluted appropriately in phosphate buffer, and analyzed by chromatography. The phosphodiester was found to be completely broken down in monomers lacking in phosphorothioate oligonucleotide. However, when urine was incubated with the oligonucleotide for 24 h, no metabolites were seen. Cosum et al. [18] used a similar approach of on-line detection of radioactive eluate from HPLC by coupling a Radiometic FLO-ONE/β Model A-

525A detector with a SE–HPLC system. In their study, a 20-mer ISIS 2105 phosphorothioate oligonucleotide (^{14}C-labeled at carbon-2 of thymidine) was used for pharmacokinetic and metabolism studies in plasma, urine, and tissues of rats. Urine or plasma was simply filtered (0.22 μm) and injected into the SAX–SE–HPLC, and collected fractions were counted by LSC using 5 ml of scintillation cocktail. HPLC followed by LSC of the eluted peaks was used by Chen and Miller [19] in the study of the disposition and metabolism of a ^{3}H-labeled 12-mer oligonucleotide methylphosphonate, following a single i.v. injection of female mice (B6D2F1) via the tail-vein. At various time points, blood, urine, and tissue/organs were obtained. Blood was collected in a heparinized tube and centrifuged at 12 000 × *g* to separate plasma. Urine samples were collected in a beaker and washed with 2 ml water. Both were stored at −20°C until analysis. Tissue digestion was performed in a scintillation vial with NCS solubilizer (12 ml g^{-1}), swirled, and heated at 50°C overnight. Decolorizer solution (1 g benzoyl peroxide in 5 ml toluene heated at 60°C, cooled and filtered) was used at 0.3 ml ml^{-1} NCS, heated for 30 min at 50°C, and cooled to room temperature. Plasma and urine were deproteinized with ethanol (1:2, v/v) and tissue was homogenized using a 3431-D 70 grinder in 4 volumes of water–ethanol (1:1, v/v). After centrifugation, the supernatant was transferred to a clean tube and evaporated under a stream of nitrogen. The residue was then reconstituted with an appropriate volume of water prior to injection into the HPLC column. Levels of radioactivity were determined in a Beckman LC8000 LSC by mixing suitable aliquots of plasma, urine, or tissue digest solution with 15 ml of scintillation cocktail. The eluent from the HPLC was also counted in the same way. The concentration of 12-mer in the samples was calculated from the specific activity of the oligonucleotide. No degradation of the 12-mer in plasma was found even 2 h post injection, whereas other organs (kidney, lung, liver and spleen) homogenate apparently produced 11-mer but no smaller metabolites.

Porta et al. [20] used a combination of three methods for the detection of DNA salt. Defibrotide is a polydeoxyribonucleotide sodium salt obtained by the controlled depolymerization of mammalian DNA. Defibrotide was injected (i.v.) into male rabbits, and blood samples were withdrawn before and after injection at different time intervals. Plasma was obtained after centrifugation and drug content was measured using (a) colorimetric method, (b) HPLC (SE type) and (c) agracose gel electrophoresis. They used trypsin digestion of plasma after incubation at 70°C for a few minutes until the plasma coagulated. Diluted sample with running buffer was injected into the HPLC.

Pneumatic-nebulization technique in ion-spray spectrometry, for the determination of molecular weights of a 14-mer synthetic deoxyribonucleotide has been demonstrated by Covery et al. [21]. The quantity of sample used was in the pmol range, achieving a sensitivity of ± 1 Da. A micro-HPLC syringe was used to deliver the sample at flow rates of 2–4 μl min^{-1}. Samples can be analyzed directly by the introduction of small quantities of the material, and on-line connection to separation and electrophoresis techniques such as HPLC and capillary-zone electrophoresis (CZE) is possible. HPLC–CE coupled to API-mass spectrometry for the detection of complex and trace unknown compounds has been demonstrated by Henion et al. [22].

4. Integrated studies with biological systems

In an effort to study the pharmacokinetics and toxicology of a number of synthetic oligonucleotides to modulate various gene expressions, Iversen [33] and his group [23–31] have performed pioneering research on both non-primates and primates, which has culminated into a Phase I clinical trial of a 20-mer antisense phosphorothioate oligonucleotide (OL(1)p53) for treatment of acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS) patients.

4.1. Fluorescence

Subcellular localization of FITC-labeled 27-mer phosphorothioate oligonucleotide was studied

with HeLa cells, and results were obtained by confocal microscopy [32] after finding an optimal section of the cell. It was found that the oligonucleotide concentration within cells was 100 times that of the surrounding medium. Both nucleus and other subcellular organelles showed oligonucleotide accumulation, and mitochondria exceeded the nuclear accumulation at every time point.

Hodges et al. [33] proposed the multiple fluorophore (monobromobimane (MBB)) labeling of phosphorothioate oligonucleotide, where ideally one fluorophore for each nucleotide residue is covalently attached to each sulfur in the backbone. These workers used pure phosphorothioate oligonucleotide to run on PAGE, both with and without 7 M urea, followed by post-labeling with 4 mM MBB to be viewed by an ultraviolet transilluminator at 366 nm. For quantification, the gel bands were excised and counted using LSC [33–35].

Similarly, MBB labeling was used to determine the concentration of OL(1)p53 in urine samples of patients in the Phase I clinical trial. This method produced reliable standard curves ($r = 0.997$, $n = 5$). However, the presence of a host of biological substances in the urine and plasma samples apparently produced a high background signal, obscuring the quantitative use of this method. When urine samples obtained before OL(1)p53 were treated with MBB, the fluorescence was greater than that observed for the urine samples obtained after infusion of the drug.

4.2. High performance electrophoresis chromatography (HPEC)

HPEC works on the principle of migration of molecules based on their charge:mass ratio as in any other gel-electrophoresis system, but they elute through a continuous polyacrylamide tube gel column (generally 2.5×50–100 mm). This method can be used to identify each band migrating through the column. The method entails running the migrating band past the gel column and detection by UV-detector.

The biodistribution of 20- and 27-mer oligonucleotide was studied in rats and mice injected for 1–3 days [1]. Liver tissue was digested with proteinase K, followed by phenol extraction and analysis by HPEC. The kidney and liver were sites of oligonucleotide accumulation, exceeding that of the plasma concentration. The majority of the oligonucleotide recovered was equivalent to authentic starting material. In a similar experiment, ^{35}S-labeled 27-mer anti-rev was injected (i.p.) into male C57/bl mice and adult male rats, and plasma was examined by LSC. The oligonucleotide was also extracted and examined by HPEC. The HPEC analysis indicated that a 50 mg kg^{-1} dose could result in a maximum plasma concentration of 5 μM. the oligonucleotide was more than 70% intact, with no $n+1$ or $n-1$ material detected.

All experiments described in the following section were performed on urine and plasma samples obtained from human subjects in the Phase I clinical trial of the antisense oligonucleotide, OL(1)p53 [23]. Urine (500Fl) was treated with 20 mM $CdCl_2$ to precipitate oligonucleotide. Following incubation at room-temperature for 10 min, the sample was centrifuged ($9055 \times g$, 5 min), the supernatant discarded, and the pellet was dissolved in 3.5 M ammonium acetate. The sample was then treated for 10 min with 4 volumes of *n*-butanol, and spun as before to obtain the lower aqueous layer, which was dried in a vacuum drier. The dried pellet was resuspended in 100 μl sterile water to load on the HPEC column. The recovery of the oligonucleotide from urine was 26–40% by this method. Detection of the oligonucleotide peak was based on the retention time [24].

Certain limitations were noted for the HPEC method. First, the retention time of standard oligonucleotides (20-mer and 40-mer) showed a positive correlation $r = 0.927$ ($n = 45$) and $r = 0.961$ ($n = 19$), respectively, with the electrophoresis run-time of the tube gel column. This obviously posed a problem in using retention times to identify the oligonucleotide peak. Second, since the migrating-peaks were eluted in $5 \times$ TBE (a high-salt buffer), the peaks could not be further examined by 5′- or 3′-end DNA labeling followed by autoradiography. If other methods of desalting (i.e. sieve-chromatography or dialysis) were to be resorted to, further handling would cause loss and degradation of the oligonu-

cleotide. Third, the loading volume of sample on the HPEC tube-gel could not exceed 20–30 μl (for standard blocks of the HPEC). Hence, if a concentrated sample was loaded within this loading volume, the low sensitivity (more than 2.0 absorbance unit) causes a loss of detection of smaller peaks (and oligonucleotide metabolites). Fourth, analysis of plasma samples on PHEC was not possible. Even though the plasma was 10 times diluted, the detection cell (≈ 2 μl) gradually blocked during the electrophoresis run time. This was probably due to the constant build up of plasma constituents in the tubing and cell walls. Although HPEC was found to have good resolution, detection sensitivity and reproducibility, it required constant monitoring, careful quantification, and suitable calculation methods to determine oligonucleotide in biological samples.

4.3. Radiolabeling

4.3.1. LSC

^{35}S-labeled 27-mer phosphorothioate oligonucleotides was studied for cellular uptake and nuclear localization in HeLa cells [32]. The cells were incubated with radioactive oligonucleotide, and then the nucleus, mitochondria and membrane/cytosol were fractionated by differential centrifugation. It was found that the oligonucleotide concentration within the cells was 100 times that of the surrounding medium.

An in vivo study of the pharmacokinetics and tissue distribution of radiolabeled oligonucleotides has been conducted in order to modulate gene expressions in different animals. A uniformly ^{35}S-labeled 27-mer phosphorothioate oligonucleotide complementary to the rev gene of HIV was studied in mice, rats, and rabbits [32]. 21–40-mer, Uniformly ^{35}S-labeled antisense oligonucleotides were injected into adult male rats both by i.v and i.p. routes. Three ^{35}S-phosphorothioate oligonucleotides behaved in a similar manner, indicating that the length and composition did not substantially influence plasma clearance. Using LSC, it was found that the oligonucleotide was completely eliminated predominantly as the parent compound in urine over 3 days. 5′-cholesteryl-conjugated phosphorothioate oligonucleotides with sequence complementary to rat CYP2B1 mRNA were evaluated for their pharmacokinetic properties, toxicity, and ability to modulate CYP2B1 expression in vivo in adult male Sprague-Dowley rats [36]. 20–22-mer Phosphorothioate-oligonucleotides were 3′-end labeled by [γ^{35}-S]dATP and the rats were injected i.p. About 250 μl blood was drawn by tail or retro-orbital bleeding at specified times, microcentrifuged at $15\,000 \times g$ for 8 min, and 50 μl plasma was counted using LSC. In a separate study of pharmacokinetics and tissue distribution of a double stranded phosphorothioate oligonucleotide, a ^{35}S-labled 25-base pair long sequence identical to the CYP1A1 negative regulatory element (NRE), was administered (i.p., 110 nmol or i.v., femoral vein, 75 nmol). Blood was drawn from the retro-orbital vein of the eye into microfuge tubes, centrifuged (10 min), and 50:1 plasma was assayed for ^{35}S-labeled 25-base pair long sequence identical to the CYP1A1 NRE. Blood was drawn from the retro-orbital vein of the eye into microfuge tubes, centrifuged (10 min), and 50:1 plasma was assayed for ^{35}S-activity using LSC. On day 4, animals were sacrificed and organs (i.e. brain, liver, lung, heart, kidney and tests) were weighed and 0.2–0.4 g of each was solubilized in 1.5 ml NCS tissue solubilizer for 2 days. 4.5 ml Of ACS scintillation cocktail was added, and the mixture counted using LSC.

One patient received uniformly ^{35}S-labeled phosphorothioate oligonucleotide, OL(1)p53 at a dose of 0.25 mg kg^{-1} h^{-1} for 10 days. The urine samples collected during this period were used to determine the oligonucleotide recovery by different processing methods (i.e. CdCl precipitation, phenol extraction, SAX processing followed by C18HPLC, filtration followed by HPLC) besides direct liquid scintillation counting. The SAX column (12 ml capacity) was preconditioned with 10 mM phosphate buffer (pH 7.5). 5 ml Of phenol–water–chloroform extracted (1:1:1, v/v/v) urine was incubated with 8 M urea at 37°C for 1 h before loading on the preconditioned SAX column. After washing with 10 ml phosphate buffer, the column was eluted with 1 M NaCl. The eluate was counted using LSC before and after further purification on C18 HPLC. the SAX

column bound ≈ 54% oligonucleotide, and 50% of it was eluted. More than 90% full size of oligonucleotide was recovered by using the combination of SAX–HPLC purification techniques

4.3.2. PAGE

Oligonucleotide was recovered from blood containing chronically infused 20-mer phosphorothioate, an antisense to p53 of the Rhesus monkey [1]. the recovered oligonucleotide showed predominantly full size authentic starting material as determined by gel electrophoresis. Some smaller and larger metabolites were also found on the gel, the composition of which was not identified.

Using the urine and plasma samples of patents in the Phase I clinical trial of OL(1)p53, the oligonucleotide was determined by PAGE analysis. The samples were processed by different methods (i.e. (a) filtration (0.22 fm), (b) phenol extraction (1:1, v/v), (c) CdCl (20 mM) precipitation, (d) SAX processing) and all these were followed by reverse-phase HPLC, before labeling the HPLC fractions at the 5′-end using [γ^{32}P]dATP and T4 polynucleotide kinase. The PAGE analysis was done using 20% polyacrylamide gel, with 3.5 M and 7 M urea followed by autoradiography and densitometry. SAX purification followed by RP-HPLC yielded maximally pure (more than 80%) full size oligonucleotide.

4.4. HPLC

Both urine and plasma samples could be evaluated either by the cadmium precipitation method or by phenol extraction (1:1, v/v), using regular C18 reverse-phase chromatography. For a urine sample, phenol–water–chloroform was added (1:1:1, v/v/v) to urine, vortexed briefly, and incubated at room-temperature for 10–20 min before centrifuging (9055 × *g*, 5 min). The top aqueous layer was analyzed by C18 HPLC (4.6 × 250 mm, 80 D, 5 Fm). Plasma samples were diluted 1:3 in sterile water before extracting with the phenol–water–chloroform reagent [24].

Experience with the determination methods of oligonucleotide in biological samples (urine and plasma) indicates that both should be processed freshly or stored at 4°C with 0.05% sodium azide until they are analyzed. Using phenol-extraction followed by the C18 HPLC method, both types of biological samples can be quantitatively analyzed. However, it is essential to cross-check the validity of the calculations based on the integrated chromatograms using 5′- or 3′-end DNA labeling and PAGE analysis followed by densitometry. This is because the full size oligonucleotide and its metabolites in the biological samples do not always elute in HPLC at retention times equivalent to those of standard oligonucleotide and its metabolites. It was found that even those peaks eluted by HPLC at different retention times were both parent oligonucleotide and its metabolites. This is obviously due to binding of the oligonucleotide and its metabolites with the other constituents present in the biological samples.

5. Conclusion and integrated summary

There seems to be an enormous potential for synthetic oligonucleotides to emerge as a new generation of gene specific therapeutic drugs. A few excellent review on various aspects have dealt with studies conducted to understand the therapeutic potential of this drug class [5,23–25]. A few of the synthetic oligonucleotides are already in clinical trials (Phase I and II) for treatment of HIV-1 and leukemia. Over the past decade or two, investigators have developed many methods to qualify and quantify these oligonucleotides and their metabolites in cell free systems, cell culture, and biological samples obtained from animals and human subjects. Both mechanisms of receptor mediation and endocytosis have been proposed for internalization of the input oligonucleotides. Both crude and processed biological samples (especially plasma and urine) have been used to qualitatively and quantitatively analyze the oligonucleotide by techniques such as fluorescence, radiolabeling, or HPLC. Measurement of oligonucleotide using direct fluorescence or radiolabeling has some inherent problems, such as poor labeling at either the 3′- or 5′-ends, quenching by cellular or biological constituents, processing conditions which affect the labeled moiety, detection and quantification strategies followed, etc. In our

experience, careful processing of biological samples (especially plasma and urine) by phenol extraction followed by reverse-phase HPLC produced 85–95% recovery and a sensitivity of 30 pmol. Anion exchange HPLC can be employed for reasonable resolution of 3–5 bases of the input oligonucleotides. The coupling of capillary electrophoresis or HPLC with mass spectrometry would be an ideal combination for the precise and reliable analytical measurement of such oligonucleotides. However, HPLC followed by post-radiolabeling, autoradiography, and densitometry would be an ideal alternative for the quantitation of oligonucleotides in biological samples for any laboratory with reasonable resources.

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