Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 129–139
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development and validation of a sensitive, specific, and rapid hybridization-ELISA assay for determination of concentrations of a ribozyme in biological matrices

Patricia Brown-Augsburger[a,*], Xin Min Yue[a], Jennifer A. Lockridge[b], James A. McSwiggen[b], Dimple Kamboj[a], Kathleen M. Hillgren[a]

[a] *Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Drop Code 0720, Indianapolis, IN 46285, USA*
[b] *Sirna Therapeutics Inc., 2950 Wilderness Place, Boulder, CO 80301, USA*

Received 29 May 2003; received in revised form 25 July 2003; accepted 25 July 2003

Abstract

Ribozymes are RNA or modified RNA polymers capable of catalyzing cleavage reactions in target strands RNA, and are under development as human therapeutics. Previous methods used for quantitation of nucleic acid polymers in serum or plasma required extraction of the polymer followed by capillary electrophoresis, HPLC, or gel electrophoresis. These methods are time consuming and lack sensitivity. A bioanalytical method has been developed that does not require extraction of the ribozyme analyte from serum. This technique relies on hybridization of the ribozyme molecule to two complementary biotin and digoxigenin labeled oligonucleotide probes. Serum containing the ribozyme is mixed with the labeled probes, and the mixture is heated at 75 °C for 5 min to disrupt the ribozyme secondary structure. Samples are then cooled to permit probe annealing and are added to a streptavidin-coated 96-well plate. The bound complex is detected with an anti-digoxigenin alkaline phosphatase (AP) conjugate using PNPP (*p*-nitrophenyl phosphate) as a substrate. The amount of colored product is measured on a microtiter plate reader at a wavelength of 405 nm. Concentrations of unknown ribozyme samples are estimated based on a standard curve (0.37–270 ng/ml) prepared in serum. The validated lower and upper limits of quantification are 5.0 and 120 ng/ml, respectively. The assay can be completed in approximately 5 h and does not require extraction procedures or electrophoretic/chromatographic separation. It is therefore a simple, sensitive and rapid technique. This assay has been validated and has been used for quantitation of serum levels of the HEPTAZYME™ ribozyme in mouse, monkey, and human pharmacokinetic studies.

Keywords: Hybridization; ELISA; Ribozyme; Oligonucleotide

1. Introduction

HEPTAZYME is a synthetic ribozyme with in vitro and cell culture activity against the plus-strand RNA of hepatitis C virus (HCV). The ribozyme specifically targets a highly conserved site within the internal ribosome entry site (IRES), which is located within the

* Corresponding author. Tel.: +1-317-277-7029; fax: +1-317-276-4218.
E-mail address: brown_aug@lilly.com (P. Brown-Augsburger).

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.japna.2003.07.002

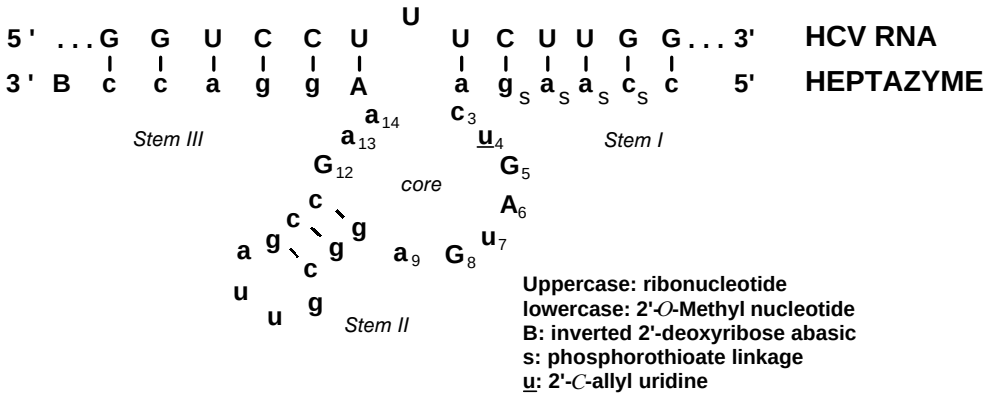

Fig. 1. Diagram of HEPTAZYME. The upper sequence is the complementary region of RNA that is the site of HEPTAZYME binding. The lower sequence is the HEPTAZYME molecule, which contains both native and chemically modified ribonucleotides. "B" is an inverted abasic nucleotide; lower case letters indicate the presence of a 2′O-Me modification; "u" indicates the presence of a 2′C-allyl uridine; "s" indicates the presence of a phosphorothioate linkage. Uppercase letters indicate unmodified native ribonucleotides. The uppercase GA sequence in the HEPTAZYME molecule is a site of potential metabolism due to the presence of adjacent unmodified ribonucleotides.

5′ untranslated region (UTR) of HCV. HEPTAZYME consists of 33 natural or chemically-modified ribonucleotides (Fig. 1). Following hybridization of ribozyme to target, the complimentary target RNA species is cleaved, resulting in a decrease in target expression.

Previous bioanalytical methodology used in the determination of ribozyme or antisense oligonucleotide concentrations required extraction of the drug from the biological matrix followed by chromatography, electrophoresis, or capillary gel electrophoresis for quantitation [1–6]. These methods are time-consuming and lack sensitivity; plasma lower-limits of quantitation typically range from 50 to 100 ng/ml.

This report describes a sensitive method for quantifying a ribozyme in serum samples that does not require extraction of the analyte from the biological matrix. The method described in this report takes advantage of the specific high-affinity binding interactions that occur during nucleotide base pairing. Sample pretreatment, hybridization stringency, and recovery were optimized to maximize sensitivity and specificity of the assay while avoiding analyte extraction. This method was validated in cynomolgus monkey, mouse, and human serum. Subsequently, the hybridization-ELISA was successfully employed to analyze samples from mouse and primate preclinical studies, and was also used to analyze samples from a human clinical study designed to evaluate the safety and pharmacokinetics of HEPTAZYME.

2. Materials and methods

2.1. Reagents

HEPTAZYME was provided as lyophilized material by Sirna Therapeutics Inc. Concentrations of solutions used as analytical standards or for dosing in animal studies were determined based on optical density at 260 nm. Streptavidin-coated microtiter plates (transparent, 96-well) and anti-digoxigenin alkaline phosphatase conjugate (anti-Dig-AP FAB fragment) were purchased from Roche Molecular Biochemicals, Inc. (Indianapolis, IN). Cluster tube eight-well strips were obtained from Fisher Scientific (Pittsburgh, PA). Phase lock gel tubes were obtained from Eppendorf 5′ (Boulder, CO). DNA oligonucleotides used to capture and detect the ribozyme were custom synthesized by Sigma–Genosys (The Woodlands, TX). Each DNA oligonucleotide was complimentary to half of the sequence of the ribozyme molecule. Oligonucleotides were modified with the addition of a 3′ biotin to oligo 1 (5′-gcctcatcagtcttgg-Biotin-3′), and a 5′ digoxigenin on oligo 2 (5′-digoxigenin-aaggtcctttcggctaac-3′). Additionally, two terminal deoxyadenosine nucleotides

were placed on the 5′ end of oligo 2 to improve binding by reducing steric hindrance. ImmunoPure PNPP (*p*-nitrophenyl phosphate) substrate and 5× diethanolamine substrate buffer were purchased from Pierce (Rockford, IL). Human serum was purchased from Western States Plasma (Oceanside, CA). Tris base, magnesium chloride, sodium dodecyl sulfate, EDTA (ethylenediamine tetra-acetic acid, dipotassium salt), and sodium chloride were RNAse-free molecular biology grade from Sigma Chemical Co. (St. Louis, MO). Diethyl pyrocarbonate (DEPC) and sodium hydroxide were reagent grade from Sigma Chemical Co. Water used in preparation of stock solutions or buffers (except ELISA wash buffer) was pre-treated with DEPC to remove RNAse contamination.

2.2. Hybridization-ELISA procedure

For preparation of standards and control samples, lyophilized HEPTAZYME reference standard material was suspended in DEPC water to achieve a concentration of 1 mg/ml. For calibration standards, this stock was then serially diluted into serum (human, mouse, or monkey) to concentrations of 270, 90, 30, 10, 3.3, 1.1, and 0.37 ng/ml. Control samples were diluted in serum at concentrations of 120, 80, 40, and 5 ng/ml.

Each of the DNA oligonucleotides used to capture and detect HEPTAZYME was resuspended at a concentration of 100 μg/ml in DEPC water. Oligonucleotides were then further diluted to a concentration of 1 μg/ml in hybridization buffer (20 mM Tris–HCl, 50 mM NaCl, 0.5 mM $MgCl_2$, pH 7.4). Serum dilution buffer (120 μl of 20 mM Tris, 2.5% SDS, and 0.5 mM $MgCl_2$) was added to cluster tubes, followed by addition of 30 μl of standard, control, or sample. A volume of 75 μl of each of the 1 μg/ml oligonucleotides was added to the diluted serum, after which the tubes were covered and heated at 75 °C for 5 min in a water bath. After heating, the tubes were removed from the water bath and cooled for approximately 5 min at room temperature. A 100 μl volume of the hybridization mix was transferred to duplicate wells of a 96-well streptavidin microtiter plate. Plates were sealed with an adhesive plate cover and placed on a rotating shaker (∼450 rpm) at 37 °C for 2 h. Non-bound material was removed with 5 × 5 washes using an EL404 plate washer (20 mM Tris Base, 150 mM NaCl, 0.1% Tween-20, pH 10.0). The anti-Dig-AP antibody was diluted to 250 mU/ml in hybridization buffer, and 100 μl was added to each well. The plate was then incubated for 1.5 h at 37 °C with shaking at ∼450 rpm. Following the antibody binding step, the plate was again washed as described above. PNPP substrate was prepared with ethanolamine buffer as described by the manufacturer, and 100 μl was added to each well. Development of the 270 ng/ml standard was monitored at 405 nm. When this standard reached an optical density of approximately 2.5, development was stopped by the addition of 25 μl of 20% EDTA to each well. Plates were read at 405 and 485 nm, and the 485 reference readings were subtracted from the reading at 405. Data were analyzed using a four or five parameter logistic algorithm (StatLia, Brendan Scientific, Carlsbad, CA).

2.3. Capillary gel electrophoresis

Analysis of study samples by capillary gel electrophoresis was performed as follows. In brief, 100 μl of plasma was mixed with 20 μl of 10% NP-40, 10 μl of internal standard, and 400 μl of Tris buffer (10 mM, pH 8). Samples were heated at 95 °C for 15 min and transferred to Phase Lock Gel I Light (Eppendorf 5′, Boulder CO) extraction tubes containing 100 μl phenol/chloroform/isoamyl alcohol (25:24:1). Samples were vortexed and centrifuged, and then an additional 200 μl chloroform/isoamyl alcohol (24:1) was added to the tubes. Tubes were vortexed and centrifuged, and the aqueous phase was evaporated using a Zymark TurboVap. Samples were reconstituted with water and TEAA, applied to a SPEC PH 3 ml/15 mg cartridge, and eluted with water/MeOH (1:1). Samples were evaporated, then reconstituted with water and dialyzed on a 0.025 μm floating desalting floating membrane filter (Millipore, Billerica MA) for 2 h. Capillary gel electrophoresis of samples was performed as described [2]. This assay had a validated lower limit of quantitation of 105 ng/ml.

2.4. Nonclinical sample analysis

Nonclinical studies were conducted to evaluate the pharmacokinetics of HEPTAZYME following subcutaneous administration. In Study A and Study B, three male and three female cynomolgus monkeys (*Macaca fascicularis*; Charles River, Mauritius)

were administered 2 mg/kg HEPTAZYME as a subcutaneous injection. HEPTAZYME solutions were prepared in normal saline. Samples were collected as plasma (Study A) or serum (Study B). In Study A, plasma concentrations were determined by capillary gel electrophoresis, as described above (lower limit of quantitation of 105 ng/ml). In Study B, serum concentrations of HEPTAZYME were determined using the validated hybridization-ELISA assay (lower limit of quantitation of 5 ng/ml). All studies were conducted in accordance with local Animal Care and Use Guidelines, and were approved by local committees.

3. Results

HEPTAZYME consists of 33 natural or chemically modified ribonucleotides (Fig. 1). The primary structure of the HEPTAZYME molecule contains adjacent unmodified ribonucleotides in the core region of the molecule. Because these nucleotides had not been chemically modified to enhance resistance to nucleases, this site was potentially more metabolically labile than positions containing ribonucleotides that had been modified to increase metabolic stability. The products of cleavage at this site would be a 9-mer and 24-mer. Because of the size of this putative 24-mer metabolic product, it had potential for significant cross-reactivity in a hybridization-based assay. The assay was optimized through alteration in hybridization stringency (temperature and salt content) to minimize cross-reactivity of the 24-mer putative metabolite, maximizing selectivity for parent molecule. Cross-reactivity of the $n-1$ and $n-2$ species (possible synthetic impurities with biological activity) was also evaluated.

3.1. Assay design

A schematic of the general assay design is shown in Fig. 2. Biotinylated and digoxigenin-labeled oligonucleotides were designed to be complementary to the HEPTAZYME sequence. Standards were added to diluent buffer in microtiter cluster tubes. Subsequently, hybridization buffer containing the two labeled oligonucleotides was added, and the entire mixture was heated to 75 °C for 5 min to disrupt the secondary structure in the HEPTAZYME molecule

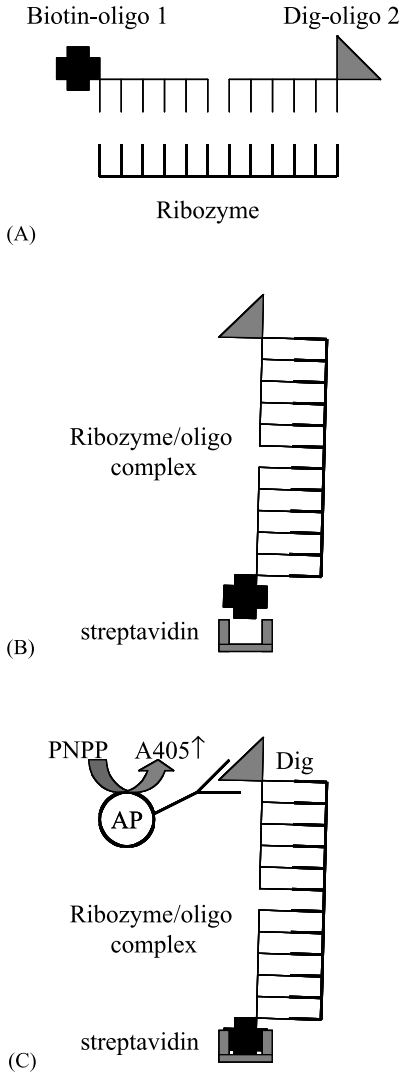

Fig. 2. Schematic of the hybridization-ELISA setup. In panel A, the denatured ribozyme is mixed with biotin- and digoxigenin(Dig-)-labeled oligonucleotides, and binding occurs by complementary base pairing. In panel B the ribozyme–oligonucleotide complex is transferred to a microtiter plate coated with streptavidin (SA). The biotin-labeled complex binds to the streptavidin, and non-bound material is washed away. In panel C, an alkaline phosphatase (AP) conjugated antibody against digoxigenin is added to the microtiter plate. The alkaline phosphatase catalyzes transformation of the PNPP substrate to a yellow color, which is detected using a microtiter plate-reader.

(Fig. 2A). The assay mixture was then transferred to streptavidin-coated microtiter plates for hybridization and for binding of the oligonucleotide–ribozyme complex to the solid phase (Fig. 2B). After washing to remove non-bound materials, the anti-Dig-AP and the substrate PNPP were used to generate a response which was measured at 405 nm using an ELISA plate reader (Fig. 2C).

3.2. Denaturation temperature

Since HEPTAZYME normally exists in a tightly bound stem-loop configuration, it was necessary to denature this self-binding to permit hybridization of the capture and detection oligonucleotides. Initial attempts to denature the molecule in serum at 95 °C for 5 min caused aggregation and precipitation of serum proteins. The resulting precipitate could not be removed effectively by either centrifugation or filtration. In subsequent attempts, it was found that heating serum samples at 75 °C for 5 min was apparently sufficient to unfold the secondary structure of the ribozyme. Under these conditions, there was no formation of protein precipitates. This denaturation condition was therefore adopted for subsequent work. As demonstrated by the use of control samples containing known amounts of HEPTAZYME (Table 1), these denaturation conditions were sufficient to permit accurate recovery of HEPTAZYME diluted in monkey serum. Similar results were achieved in mouse and human serum (not shown).

3.3. Salt concentration

In initial experiments to evaluate the feasibility of the assay format, hybridization was performed at room temperature in a solution containing a final concentration of 150 mM NaCl. Fig. 3 shows the effect of decreasing salt concentration on the assay response of standards prepared in human serum with full-length HEPTAZYME or the 24-mer putative metabolite. Hybridization buffers, prepared with sodium chloride concentrations ranging from 25 to 300 mM, were tested in the assay. The results from 50 to 300 mM are shown to illustrate the effect of salt concentration on the specificity of the assay. Note that the final sodium chloride concentration of the hybridization reaction is half of the concentration in the hybridization buffer due to mixing with the diluted serum sample on the ELISA plate. Results from 25 mM NaCl were comparable to those at 50 mM, and are not shown. For full-length HEPTAZYME, assay response only decreased slightly as salt concentrations decreased. However, for the 24-mer metabolite, assay response was significantly diminished by reducing NaCl from 300 to 50 mM. This experiment provided evidence showing that the HEPTAZYME/24-mer response ratio could be maximized by reducing the sodium chloride concentration to 50 mM in the hybridization buffer, resulting in a final sodium chloride concentration of 25 mM in the hybridization reaction.

3.4. Hybridization/binding temperature

An additional variable that could affect the hybridization specificity was the binding temperature. Initially, binding of oligonucleotides to the ribozyme and binding of the anti-digoxin-AP conjugate were performed at room temperature. Because the melting temperature (T_m) is generally higher when there are more base pairs formed, it was expected that increasing the temperature at which binding occurred

Table 1
Cynomolgus monkey serum validation data: intra-assay and inter-assay precision and accuracy

Control sample concentration (ng/ml)	Intra-assay[a]			Inter-assay[b]		
	Mean (S.D.)	%CV	%Accuracy	Mean (S.D.)	%CV	%Accuracy
5.0	5.3 (0.3)	6.3	105.0	5.1 (0.3)	6.2	102.5
40.0	41.8 (2.3)	5.6	104.6	40.8 (2.5)	6.2	102.0
120.0	121.0 (4.8)	4.0	100.8	118.9 (5.6)	4.7	99.1

%CV: percent coefficient of variation; S.D.: standard deviation.
[a] Number of replicates within run = 6.
[b] Number of assay runs = 4.

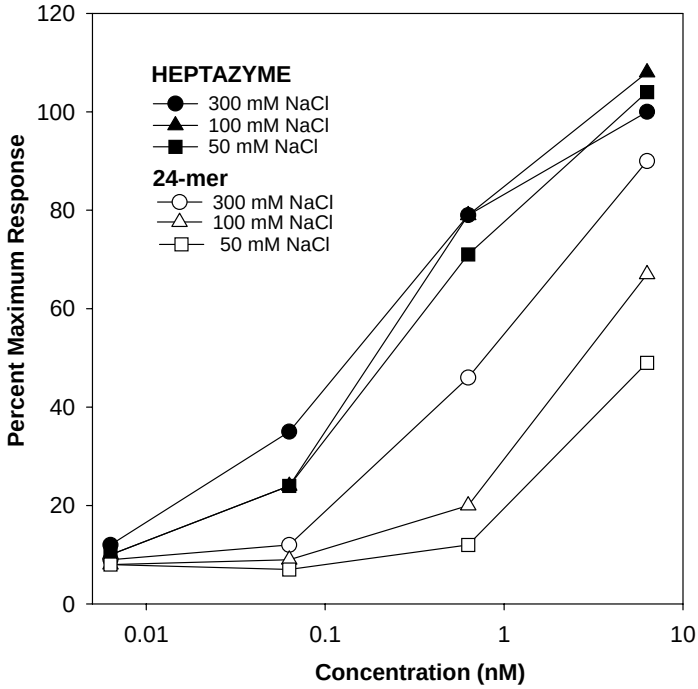

Fig. 3. The effect of differing salt concentration on metabolite response in hybridization-ELISA. The hybridization-ELISA was performed with full-length HEPTAZYME or the 24-mer putative metabolite using hybridization buffers containing NaCl concentrations ranging from 25 to 300 mM. Results from 50, 100, and 300 mM NaCl concentrations are shown. Open symbols represent the HEPTAZYME molecule, while closed symbols represent the 24-mer.

would reduce cross-reactivity of truncated fragments. An experiment was performed examining the effect of conducting binding incubations at room temperature or 37 °C, in which the responses of full-length HEPTAZYME and 24-mer fragment were compared. Both the initial 2 h incubation on the streptavidin plate and the subsequent 1.5 h incubation with the anti-digoxigenin AP conjugate were performed at the same temperature (room temperature or 37 °C). Fig. 4 shows response curves for HEPTAZYME and 24-mer fragment generated at room temperature and 37 °C. While there was little alteration in the response curves generated at either temperature for the full-length HEPTAZYME molecule, response for the 24-mer was significantly decreased at 37 °C relative to room temperature. This modification of incubation temperatures, in combination with determination of an appropriate salt concentration for the hybridization buffer, resulted in less than 5% cross-reactivity of the 24-mer at concentrations in the validated range of the assay. The 31-mer (3′ and 5′ $n-2$) and 32-mer (3′ and 5′ $n-1$) species demonstrated at least 75% cross-reactivity in the assay.

3.5. The effect of detergent on recovery of spiked controls

During evaluation of assay performance in monkey and mouse serum, it was determined that there was lack of recovery in the assay following addition of HEPTAZYME into some pooled lots or individual samples of serum. It was hypothesized that non-specific binding of HEPTAZYME to serum components was responsible for the variable recovery in the animal sera evaluated, and that addition of detergent might dissociate the interactions and improve recovery. Tween-20, Triton-X-100, and sodium dodedcyl sulfate (SDS) were evaluated for their ability to improve analyte recovery when added to the hybridization buffer. It was found that only SDS was signifi-

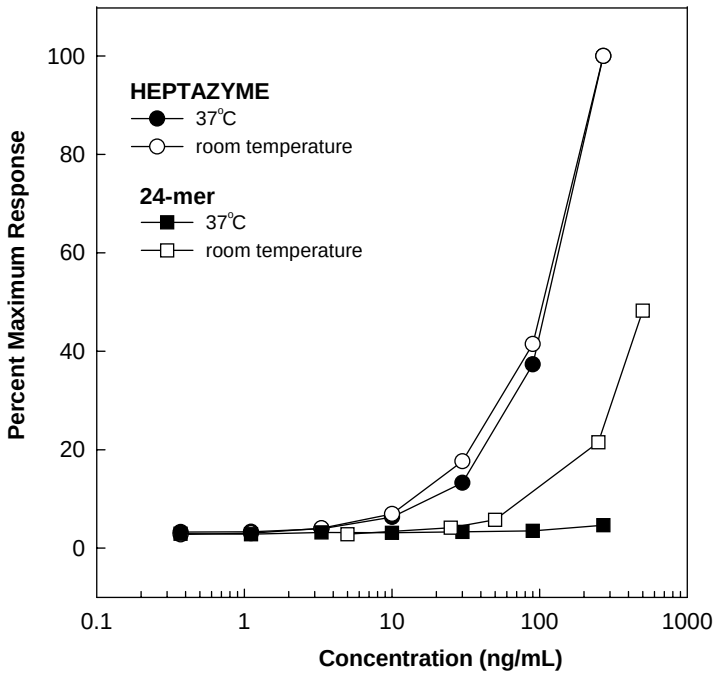

Fig. 4. The effect of incubation temperature on hybridization-ELISA specificity for HEPTAZYME vs. the putative 24-mer metabolite. The hybridization-ELISA was performed as described in Section 2, using the hybridization buffer containing 50 mM NaCl. All incubations were performed either at room temperature or at 37 °C, as indicated.

cantly effective at improving HEPTAZYME recovery and reducing variability of recovery. Table 2 shows the results of an experiment in which HEPTAZYME was spiked in monkey serum samples (either individual samples or pooled lots) at 80 ng/ml. These control samples were assayed versus a standard curve prepared in serum pool 1. As shown in Table 2, in the absence of SDS, recovery of spiked controls was in many cases low and highly variable. However, when the same samples were assayed in the presence of SDS (1% final in the hybridization mixture), recovery of spiked controls ranged from 97 to 120% of theoretical. A similar observation made in mouse sera, where the addition of SDS in the hybridization mixture also resulted in improved recovery control samples. Low and variable recovery was not observed with HEPTAZYME spiked into a variety of human serum samples and assayed in the absence of SDS. However, the methodology for all matrices was modified by adding SDS to the hybridization buffer. In human and non-human primate serum and plasma, SDS may have the added advantage of lysing enveloped virus particles.

Table 2
Percent recovery of HEPTAZYME in cynomolgus monkey serum measured by hybridization-ELISA with and without SDS

Serum[a]	%Accuracy − SDS	%Accuracy + SDS
M-1	33	115
M-2	25	116
M-3	43	120
M-4	34	118
M-5	26	105
M-6	51	112
M-7	59	115
M-8	53	107
Pool 1	84	106
Pool 2	26	97
Pool 3	9	103
Pool 4	36	103

[a] Sera from individual animals (M-1 through M-8) or pools of monkey serum (pools 1–4) were spiked with 80 ng/ml of HEPTAZYME. Samples were assayed with or without the addition of 2.5% SDS to the sample dilution buffer resulting in 1% final concentration in the hybridization mixture.

Table 3
Standard curve data in cynomolgus monkey serum: average back calculated standards

Run ID	Standard concentration (ng/ml)						
	270	90	30	10	3.33	1.11	0.37
1	277.0	86.8	30.8	9.9	3.4	1.0	0.4
2	275.5	87.4	31.0	9.9	3.3	1.1	0.4
3	284.8	86.3	30.0	10.4	3.6	0.8	0.5
4	279.0	85.7	31.3	9.8	3.4	1.1	0.4
Mean	279.1	86.6	30.8	10.0	3.4	1.0	0.4
S.D.	4.1	0.7	0.6	0.3	0.1	0.1	0.0
%CV	1.5	0.8	1.8	2.7	3.7	14.1	11.8
%Accuracy	103.4	96.2	92.4	100.0	102.9	90.1	114.9

S.D.: standard deviation; %CV: percent coefficient of variation.

3.6. Validation

This hybridization-ELISA has been validated for use in mouse, human, and monkey serum. Validation results in monkey serum are presented here, and are representative of data obtained for all species validated. Table 1 shows the overall precision and accuracy for control samples in the assay validation. This data demonstrates that within the 5–120 ng/ml range, the overall percent coefficient of variation (%CV) and %relative error (%R.E., %accuracy—100) were less than or equal to 7 and 5%, respectively. Table 3 shows the back-fit calculations for standard curve data used in validation runs, as well as the cumulative precision and accuracy of the back-fit calculations. Together, the evaluation of standard curve data and control values determined in validation demonstrates that assay was accurate, precise, and reproducible.

3.7. Stability

Storage stability was demonstrated by adding known amounts of HEPTAZYME into pools of monkey serum. Stored control samples were assayed using the hybridization-ELISA method following storage at room temperature for at least 3 h prior to analysis, at −70 °C for up to 12 weeks, or following up to three freeze/thaw cycles. Recovery of spiked controls under these conditions was within ±20% of theoretical. In human serum, HEPTAZYME stability was evaluated at room temperature for up to 4 h prior to analysis, following up to 5 freeze/thaw cycles, and under long term stability for 6 months at −20 °C and −80 °C. Again, controls in human serum demonstrated recovery within 20% of theoretical under these conditions.

3.8. Specificity

Because of the theoretical possibility that viral RNA sequences could interfere in determination of HEPTAZYME concentrations using the hybridization-ELISA method, we evaluated the recovery of HEPTAZYME spiked into HCV positive human serum. HEPTAZYME was spiked into human serum containing three different levels of HCV virus (Table 4). The hybridization-ELISA assay was performed on the spiked HCV positive controls and on blank samples (minus HEPTAZYME). No signal was detected in the blank HCV positive sera. Also, there was no apparent interference in the assay in the controls containing HEPTAZYME.

3.9. Study sample analysis

Serum samples obtained from a cynomolgus monkey pharmacokinetic study (Study B) were analyzed using the hybridization-ELISA assay described in

Table 4
Measurement of HEPTAZYME control samples prepared in HCV positive human serum

	Viral load	Blank	Theoretical concentration	
			5 ng/ml	96 ng/ml
Mean[a]	60450	BQL[b]	5.90	116
%CV		–	4.34	1.72
%Accuracy		–	118	121
Mean	178329	BQL	5.59	105
%CV		–	3.58	1.65
%Accuracy		–	112	109
Mean	603750	BQL	6.02	117
%CV		–	2.49	5.71
%Accuracy		–	121	122
Overall mean		–	5.84	113
Overall %CV		–	4.49	5.96
Overall %accuracy		–	117	117

[a] $N = 3$ determinations at each control concentration for each source.
[b] BQL, below assay quantitation limit (<2.5 ng/ml).

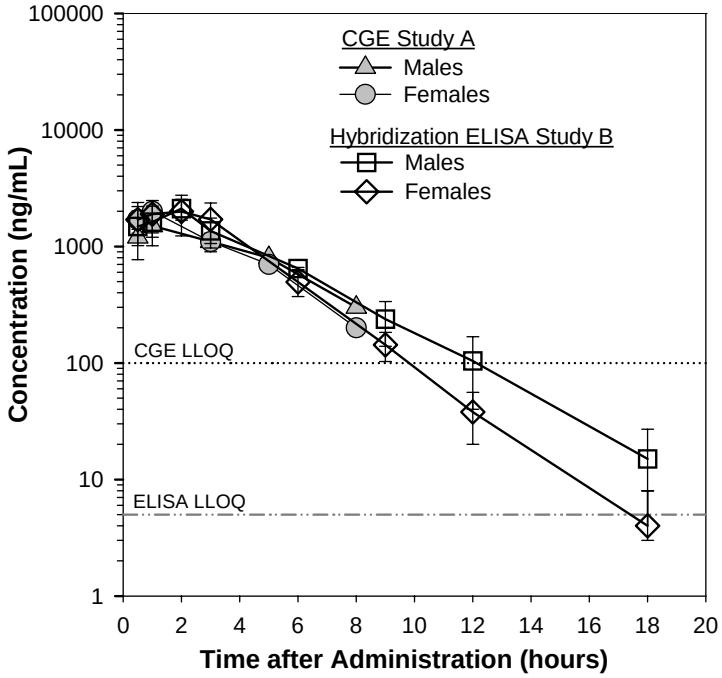

Fig. 5. Comparison of HEPTAZYME pharmacokinetics measured by CGE or hybridization-ELISA. The pharmacokinetics of HEPTAZYME in male and female cynomolgus monkeys were measured following administration of 2 mg/kg by the subcutaneous route in two separate studies. In Study A, plasma concentrations were measured by a validated capillary gel electrophoresis method. In Study B, serum concentrations were measured by validated hybridization-ELISA. Points represent mean ± standard deviation, $N = 3$ animals per group. LLOQ: lower limit of quantitation.

this paper. Results are summarized in Fig. 5, and are shown in comparison to data obtained in a previous study in which the pharmacokinetics of HEPTAZYME were evaluated using capillary gel electrophoresis (Study A). Data obtained from the CGE and hybridization-ELISA assays were similar at time points from 0 to 8 h after HEPTAZYME administration. However, since the lower limit of quantitation for the CGE assay was 105 ng/ml, no data was obtained after the 8 h time point in Study A, and there were insufficient terminal phase data points to permit determination of a half-life of elimination. In contrast, the hybridization-ELISA assay sensitivity permitted characterization of exposure through 18 h after subcutaneous administration of HEPTAZYME, and an elimination half-life of 2.3 h was determined in Study B. The improved sensitivity of the hybridization-ELISA assay therefore permitted a better understanding of the pharmacokinetic behavior of HEPTAZYME.

4. Discussion

Ribozymes are polymers of RNA or RNA analogs that can catalyze the cleavage of other RNA molecules [7]. Like antisense oligonucleotides [8], ribozymes are designed to inhibit transcription of an RNA message and ultimately prevent production of unwanted proteins or viral products. As polynucleotides like ribozymes and antisense oligonucleotides are developed as therapeutics, the need arises to measure concentrations of these drugs in blood and other biological matrices. Conventional approaches to bioanalytical quantification of polynucleotides have included HPLC, electrophoresis, or capillary gel electrophoresis [1–4,6]. Overall, these approaches have limited sensitivity (50–100 ng/ml) and are time intensive because they require extraction of the polynucleotide of interest from a biological matrix. This extraction step may contribute to the

limited sensitivity because of incomplete recovery of analyte.

The assay methodology described in this report is a novel approach to quantifying a therapeutic nucleotide-based drug, and has a number of advantages over conventional techniques. The hybridization-ELISA does not require an extraction step, which represents a major advantage in speed, cost, and recovery. It was determined that denaturation of the HEPTAZYME hairpin loop, which was necessary to create access for the capture/detect oligonucleotides, occurred at 75 °C, and that at this temperature, significant serum protein aggregation and precipitation did not occur in the diluted assay mixture. As the hybridization-ELISA assay was developed, it became apparent that recovery of analyte could vary among different matrix sources. The probable explanation for this was non-specific binding of the ribozyme molecule to components in serum. We explored the addition of detergents in hybridization mixture, and found that 1% SDS (final concentration) was sufficient to disrupt the non-specific binding and permit accurate recovery of samples spiked into serum from a variety of sources. Other detergents were evaluated and were found to be less effective in improving analyte recovery. This solution to the recovery problem permitted the use of the hybridization-ELISA without pre-extraction of samples.

In two recent publications, authors described hybridization-based assays using single oligonucleotide probes, one that was run in a competitive format and required a radiolabeled tracer [9], and a second assay using hybridization and ligation (DNA ligase) [10]. Because of the size of the HEPTAZYME molecule, we were able to use two oligonucleotides to form a "sandwich" for analyte capture and detection. This aspect of the assay design insured that small fragments of a ribozyme, which may arise as products of metabolism, would not bridge both sides of the sandwich, and would therefore be undetected. The two probe design also insured that the melting temperature (T_m) of the complex was low enough that modest changes in hybridization stringency (temperature or salt concentration) could improve assay specificity. The T_m of each oligonucleotide was approximately 56 °C, while the T_m of a single oligonucleotide that spanned the entire HEPTAZYME sequence would be approximately 73 °C. Finally, the approach employed here does not require an enzymatic step, which could add to assay variability, or the use of radioisotopes, which have the disadvantages inherent in synthesizing, tracking, and disposing radionuclides. It is unclear, however, how large the analyte oligonucleotide drug must be in order for this assay approach to be applicable. It is likely that the T_m of two very short probe oligonucleotides may be too low to permit good assay sensitivity, or that the higher T_m of very long oligonucleotides may prevent application of adjustments in hybridization conditions to limit cross-reactivity with metabolites.

The data in Fig. 5 demonstrate that a conventional CGE method and the hybridization-ELISA yielded comparable results at concentrations where CGE was sufficiently sensitive. The hybridization-ELISA sensitivity, however, was sufficient to show the terminal elimination phase of the concentration versus time profile, and thus more clearly define the pharmacokinetics of the HEPTAZYME molecule.

In conclusion, we have developed and validated an improved technique for measuring concentrations of therapeutic polynucleotides (RNA or DNA) in biological matrices. The "sandwich" format of the hybridization-ELISA assay permits the use of non-isotopic detection for signal generation. With the design of appropriate capture and detection oligonucleotides and determination of specific hybridization conditions (e.g. temperature and salt concentrations), this assay format is applicable to analysis of therapeutic polynucleotides without significant sample pre-treatment. As demonstrated by the use of this assay with nonclinical samples, greatly improved sensitivity is possible with the hybridization-ELISA assay format described here. Additional advantages include decreased analytical costs and more rapid throughput of study samples when compared to many previously applied techniques.

References

[1] S. Agrawal, J. Temsamani, Tang, J. Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 7595–7599.
[2] L. Bellon, L. Maloney, S. Zinnen, J. Sandberg, K. Johnson, Anal. Biochem. 283 (2000) 228–240.
[3] M. Gerster, J. Schewetz, H. Fritz, M. Maier, E. Bayer, Anal. Biochem. 262 (1998) 177–184.

[4] J. Leeds, M. Graham, L. Truong, L. Cummins, Anal. Biochem. 235 (1996) 36–43.
[5] J. Sandberg, C. Sproul, K. Blanchard, L. Bellon, D. Sweedler, J. Powell, F. Capulto, D. Kornbrust, V. Parker, T. Parry, L. Blatt, Antisense Nucleic Acid Drug Dev. 10 (2000) 153–162.
[6] Y.-P. Shaw, J. Fishback, K. Cundy, W. Lee, Pharm. Res. 12 (1995) 1937–1942.
[7] T. Cech, Curr. Opin. Struct. Biol. 2 (1992) 605–609.
[8] N. Dias, C. Stein, Mol. Cancer Ther. 1 (2002) 347–355.
[9] M. deSerres, M. McNulty, L. Christensen, G. Zon, J. Findlay, Anal. Biochem. 233 (1996) 228–233.
[10] R. Yu, R. Baker, A. Chappell, R. Geary, E. Cheung, A. Levin, Anal. Biochem. 304 (2002) 19–25.