A Novel Oligodeoxynucleotide Inhibitor of Thrombin. I. *In Vitro* Metabolic Stability in Plasma and Serum

Jeng-Pyng Shaw,[1] James A. Fishback,[1] Kenneth C. Cundy,[1] and William A. Lee[1,2]

Received March 27, 1995; accepted August 7, 1995

Purpose. To determine the degradation rates and pathways of GS-522, a potent oligodeoxynucleotide (GGTTGGTGTGGTTGG) inhibitor of thrombin, in serum and plasma.

Methods. A stability-indicating, anion-exchange HPLC method was developed and used to determine concentrations of GS-522 and metabolites.

Results. In monkey plasma at 2 μM or below, the degradation of GS-522 can be fit to a first-order exponential with a $k^{p}_{obs} \sim 0.01$ min^{-1}. At 3 μM and above the degradation process deviates from a monoexponential decay profile. An initial fast degradation process is followed by a slower phase with an observed rate constant equal to that observed at 2 μM and below. In monkey serum, the K_M and V_{max} are 8.4 μM and 0.87 μM min^{-1}, respectively.

Conclusions. The kinetics are consistent with an equilibrium binding of GS-522 to prothrombin in plasma (K_d = 50 nM) which saturates at GS-522 concentrations >2 μM. Compared to a scrambled sequence (GGTGGTGGTTGTGGT), with no defined tertiary structure, GS-522 is 4-fold more stable in serum. The metabolic profile in plasma is consistent with a 3′-exonuclease catalyzed hydrolysis of GS-522.

KEY WORDS: oligodeoxynucleotides; nuclease degradation; plasma stability; kinetics; *in vitro* metabolism; thrombin inhibitor.

INTRODUCTION

Oligodeoxynucleotides represent a potential new class of chemotherapeutic agents. Complementary oligodeoxynucleotide sequences can bind mRNA with great specificity and affinity, selectively inhibiting protein synthesis by what is referred to as an "antisense" mechanism (1). Additionally, oligodeoxynucleotides have been synthesized that bind in the major groove of double-stranded DNA and are capable of halting *in vitro* transcription (2). In order to exert a biological effect, both approaches require oligodeoxynucleotides that are able to permeate cell membranes and are relatively stable to extra- and intracellular nucleases. In a recent publication by Bock *et al.*, a novel *in vitro* selection and amplification technique was described that allows target molecules to be screened for binding affinity against a pool of $\sim 10^{13}$ unique oligodeoxynucleotide sequences. This technology expands the potential application of oligodeoxynucleotides to extracellular protein targets, obviating the need for cellular permeation and cytoplasmic stability. Exploitation of this screening and amplification technology resulted in the identification of a unique oligodeoxynucleotide, GGTTGGTGTGGTTGG, with a high binding affinity for thrombin (3). This 15-mer, known as GS-522, has shown potent *in vitro* and *in vivo* anticoagulant activity (4). NMR studies have revealed a novel tertiary structure for GS-522 in solution (5, 6). The structure consists of two intramolecular G-quartets connected by a phosphodiester backbone resulting in a compact and highly symmetrical structure. An anionic binding exosite on the thrombin molecule has been identified as a putative binding site for GS-522 (7). In controlled intravenous infusion experiments in the cynomolgus monkey, the prolongation of the plasma prothrombin time (PT) was proportional to the infusion rate. Upon cessation of the infusion, the *in vivo* half-life, as observed by the decrease in PT, was less than 2 min. The potent anticoagulant activity coupled with a fast *in vivo* clearance could offer distinct advantages over current heparin therapy (8). GS-522 is currently undergoing further preclinical evaluation for use as an anticoagulant in cardiopulmonary bypass.

Published preclinical pharmacokinetic studies have relied on the prolongation of PT to estimate the activity and plasma concentration of GS-522 (4). The limit of detection of this bioassay in plasma is approximately 0.3 μM and limited information regarding its specificity is available. In order to more fully characterize the metabolism of GS-522 in selected tissues and its pharmacokinetic properties, we have developed an anion-exchange HPLC method for analysis of GS-522 and its metabolites from monkey, dog, and human plasma. To understand the role plasma metabolism plays in the pharmacokinetics of GS-522, we have thoroughly investigated the *in vitro* degradation of GS-522 in cynomolgus monkey serum and plasma. The analytical method and the results of our *in vitro* metabolism studies on GS-522 are described herein.

MATERIALS AND METHODS

Chemicals and Reagents

GS-522 was prepared by solid-phase phosphoramidite chemistry on a Biosearch 8800 synthesizer. The resulting tritylated DNA was purified on a polystyrene RP HPLC column, detritylated and further purified using a C18 RP column. The purified oligodeoxynucleotide was converted to the Na^+ form by passing it through a BIORAD AG50W-X8 column. The solution was then brought to dryness on a Speed Vac and the solid stored at −20°C. The purity was greater than 97% as determined by anion-exchange HPLC. Stock solutions of GS-522 were prepared by dissolving the oligodeoxynucleotide in water and determining the concentration from the absorbance at 260 nm ($\epsilon = 1.43 \times 10^8$ AU/mol). The scrambled sequence and the 5′-truncated oligodeoxynucleotides (n − 1 . . . n − 6) were synthesized by the same procedures.

HPLC-grade water was used to prepare all stock solutions. Phenol, chloroform, sodium perchlorate ($NaClO_4$), isoamyl alcohol, Tris·HCl, and EDTA were ACS grade and obtained from standard suppliers. Acetonitrile was HPLC grade. Pooled monkey plasma and serum were obtained from Sierra Biomedical, Inc. (Sparks, Nevada). Pooled human

[1] Gilead Sciences, Inc., 353 Lakeside Drive, Foster City, California 94404.

[2] To whom correspondence should be addressed.

0724-8741/95/1200-1937$07.50/0

plasma was obtained from George King Biomedical, Inc. (Overland Park, Kansas). Dog plasma was obtained from Stanford University (Palo Alto, California). All plasma samples were stabilized with citrate (~10 mM) except where heparin is specified in the text.

HPLC Conditions

The HPLC system comprised a Model P4000 solvent delivery system with a Model AS3000 autoinjector and a Model UV1000 UV detector (Thermo Separation, San Jose, California). A NucleoPac, PA-100 (4 × 250 mm) column was used (Dionex, Sunnyvale, California). The mobile phases used were: A: 20% acetonitrile in 50 mM $NaClO_4$, 50 mM Tris·HCl, pH 8.0; B: 20% acetonitrile in 125 mM $NaClO_4$, 50 mM Tris·HCl, pH 8.0. The flow rate was 1.4 ml/min and the column temperature was maintained at 40°C by a column oven. The gradient profile was linear to 100% B in 10 min and held at 100% B for 2 min. The detection wavelength was 256 nm and the injection volume was 50 μl. Total cycle time between injections was 20 min. Data were acquired and stored by a Peak Pro data acquisition system (Beckman; Palo Alto, California).

Sample Preparation

The isolation of GS-522 from plasma was carried out using a phenol/chloroform extraction procedure similar to that described in the literature for DNA isolation (9). Plasma or serum samples (50 μl) were mixed with 50 μl phenol/chloroform and 100 μl 10 mM Tris·HCl (pH 8.0). Sample mixtures were vortexed and centrifuged in a Biofuge centrifuge (Baxter) at 20,000 g for 10 min. The aqueous phase was transferred to a clean tube and the protein pellet was extracted again with 10 mM Tris·HCl (pH 8.0). The combined aqueous phase was dried under reduced pressure at room temperature. Dried samples were resuspended with 100 μl mobile phase A (without acetonitrile) for HPLC analysis. Recovery of GS-522 from plasma or serum was determined relative to recovery from phosphate buffered saline (PBS).

Human Prothrombin Binding

The binding of GS-522 to human prothrombin (hPT) was studied in PBS. PBS, with or without 3 μM hPT added, was incubated with 0.1, 0.3, 1, 1.5, 2.0, 2.5, and 3.0 μM GS-522 at 37°C for 30 min. The incubation mixture was filtered through a 30 kD cut-off ultrafilter (Ultrafree-MC filters, Millipore) by centrifugation in a temperature controlled Eppendorf Centrifuge at 2,000 g and 37°C for 5 min. The free fraction was determined by dividing the concentration of GS-522 in the ultrafiltrate containing 3 μM hPT by the concentration GS-522 in the control ultrafiltrate without hPT. The dissociation constant (K_d) was determined by a Scatchard Plot analysis.

Plasma and Serum Metabolism

All studies were performed in 90% plasma or serum and a blank plasma or serum control was run in parallel for each experiment. Plasma or serum samples were preincubated in a shaker bath at 37°C and 100 oscillation/min for 10 min. A stock solution of GS-522 was added to the incubation tubes, mixed, and maintained at 37°C and 100 oscillation/min. Aliquots (50 μl) were withdrawn at each time point, quenched with 50 μl phenol/chloroform, and stored at −20°C. For the initial rate studies, aliquots were quenched into phenol/chloroform solutions held at −70°C in dry ice.

Data Analysis

GS-522 concentration versus time data were analyzed using the nonlinear curve fitting software PCNONLIN (10). All data were analyzed using either a mono- or bi-exponential model. The most appropriate model was selected using the Akaike information criterion (11) and by examination of scatter plots of the residual error.

RESULTS AND DISCUSSION

Analytical Method

Attempts to separate GS-522 from plasma contaminants using either reverse-phase or anion-exchange solid phase extraction cartridges were unsuccessful. Recovery of GS-522 from monkey plasma (86%) or monkey serum (84%) was achieved using the phenol/chloroform extraction described above. The recovery of the scrambled sequence from monkey serum was also 84%. Strong anion-exchange HPLC is an excellent method for separating oligodeoxynucleotides that possess a phosphodiester backbone (12). Oligodeoxynucleotides up to approximately 40 nucleotides in length are readily resolved as a function of chain length. The addition of 20% acetonitrile to both mobile phases decreased the retention time of the majority of plasma contaminants while having little effect on the GS-522 retention time, thus greatly increasing sensitivity. Figure 1 shows the separation of GS-522 and a scrambled sequence containing the same nucleotide stoichiometry (GGTGGTGGTTGTGGT) isolated from monkey plasma.

In Table I, the linearity, range, detection limit, quantitation limit, precision, and percent recovery for GS-522 from buffer, monkey plasma, and dog plasma are shown. The extinction coefficient ($\epsilon = 1.43 \times 10^8$ AU/mol at 260 nm) was estimated from the Borer's nearest-neighbor interaction rules and experimentally determined by quantitative base

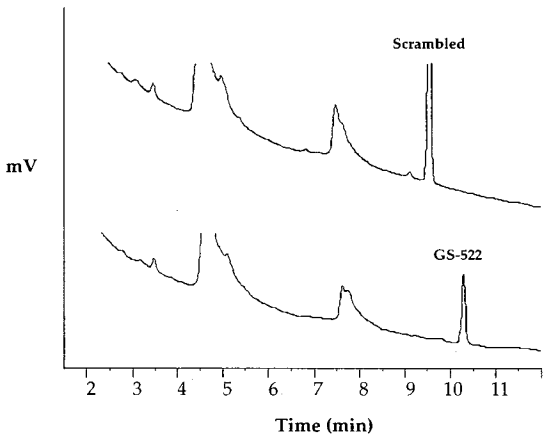

Fig. 1. Chromatograms of GS-522 (GGTTGGTGTGGTTGG) and a scrambled sequence (GGTGGTGGTTGTGGT) in monkey plasma.

Table I. Summary of Linearity, Limit of Detection, Limit of Quantitation, Reproducibility, and Percent of Recovery of GS-522 from Buffer, Monkey Plasma and Dog Plasma

	Buffer	Monkey plasma	Dog plasma
Linearity (range)	5 nM–30 μM	20 nM–30 μM	20 nM–30 μM
Detection limit	3 nM	5 nM	5 nM
Quantitation limit	5 nM	20 nM	20 nM
Precision (RSD)[a] 100 nM	1.16%	3.16%	6.3%
% Recovery[b] 100 nM	101 ± 5%	86 ± 7%	98 ± 13%

[a] Reproducibility was determined by six injections of 100 nM GS-522 from buffer or plasma.

[b] Mean ± SD recovery was determined by six separate sample preparations and injections of 100 nM GS-522 from buffer or plasma.

composition analysis (13). The stability of GS-522 in mobile phase was excellent and no special handling procedures were required. The inter-day variability of the method both in monkey and dog plasma was less than 8% in the 1 to 10 μM range.

Anticoagulant activity in plasma is customarily assessed using the PT assay. Although extremely important clinically, the low sensitivity of the assay limits its usefulness in pharmacokinetic studies and in the case of GS-522, it provides no information with regard to metabolites. The limit of detection of the HPLC assay for GS-522 is 40-fold more sensitive than the PT assay. This increase in sensitivity, while not important in a clinical setting, provides sufficient plasma concentration data to allow a detailed pharmacokinetic analysis of GS-522 in the dog and monkey.

GS-522 Binding to Human Prothrombin

Due to metabolic degradation of GS-522 in serum and plasma, the overall dissociation constants of GS-522 from plasma and serum proteins cannot be readily measured. Although GS-522 was selected from a large pool of oligodeoxynucleotides based on its binding affinity to thrombin, the homeostatic thrombin concentration is negligible (14) in plasma and would, therefore, bind only a very small fraction of GS-522 at the concentrations anticipated clinically. Using an ultra-filtration method, we have been able to measure the binding of GS-522 to prothrombin (hPT) in PBS at pH 7.4. A Scatchard plot analysis of the data resulted in a K_d value of 50 nM for the slope ($r = 0.984$) of the line. This value is 19-fold greater than the value reported for the binding of GS-522 to human thrombin (15). Since the prothrombin concentration in human plasma is reported to be 1.7 μM (16), a significant fraction of the anticipated GS-522 therapeutic dose will be bound to prothrombin.

In Vitro Metabolism in Plasma and Serum

The order of stability for the degradation of 1 μM GS-522 at 37°C in pH 7.4 buffer and in various animal plasmas is buffer > dog ~ human > monkey. No loss of GS-522 was observed in pH 7.4 buffer over this time period (1 hr). Slight variations in the processing of the individual plasmas could affect enzymatic activity and this may in part account for these differences. Figure 2 shows the effects of longer incubation times in monkey plasma at 0.1, 1.0 and 7.0 μM GS-522. The pseudo first-order rate constants determined from the fit of the data to a mono- or a biexponential decay are shown in Table II. At 0.1 and 1.0 μM, the kinetics of degradation at 37°C follow a monoexponential decay process for ≥3 half-lives over a 5 hr period, thus indicating that the nucleases in plasma are viable under these *in vitro* conditions. At ≥3 μM the quality of the fit to a single exponential diminishes due to variability in the initial time points and an apparent rapid initial degradation phase and hereafter are fit to a biexponential model. The slower constants are similar to the k^P_{obs} values observed at concentrations <3 μM and are believed to reflect the same degradation mechanism.

In order to better interpret the observed kinetic profiles in monkey plasma, the metabolism of GS-522 was also examined in monkey serum at concentrations from 0.1 to 7 μM. Prothrombin, believed to be the major binding component in plasma, is not present in serum. The kinetic data in serum can be fit to a monoexponential decay for at least 2–4 half-lives from 0.1 μM to 7 μM GS-522. The mean k^S_{obs} for the degradation of GS-522 in monkey serum is 0.090 min^{-1} (Table II). This value is approximately 11 times the average k_{obs} value calculated in plasma from 0.1 to 1.0 μM GS-522 concentration. To determine whether the difference in rate constants observed in plasma and serum reflects differences in nuclease activity or prothrombin binding in plasma, the degradation of the scrambled sequence oligodeoxynucleotide, which does not bind to prothrombin, was examined in serum and plasma. At 0.3 μM, the observed degradation rate constant for the scrambled sequence is four-fold faster in serum as compared to plasma (Table II). The potential cause of this four-fold effect is the citrate (~10 mM) used in the processing of plasma. Citrate chelates divalent metals required for activity for some nucleases and has been reported to inhibit chromosomal DNA degradation (17). Indeed, citrate does inhibit the degradation of 1 μM GS-522, however, the effect appears to reach a maximum (4–5 fold) between 10 and 20 mM citrate at pH 7.4 (data not shown). The degradation of GS-522 is 2.5 times faster in plasma treated with heparin than in plasma treated with citrate, consistent with

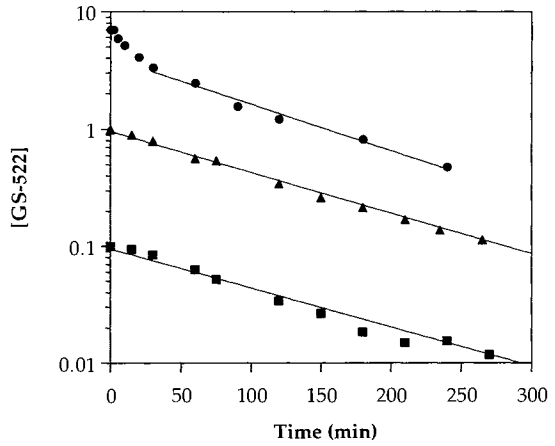

Fig. 2. Effect of concentration on the degradation of GS-522 in monkey plasma at 37°C. (■): 0.1 μM; (▲): 1 μM; (●): 7 μM.

Table II. Observed Pseudo First-Order Rate Constants for GS-522 Degradation in Monkey Plasma and Serum

[GS-522] concentration (μM)	Plasma k^p_{obs} min^{-1}	Serum k^s_{obs} min^{-1}
0.10	0.0087	0.094
0.30	0.0072	0.090
1.00	0.0094	0.093
2.00	0.0121	
3.00	0.0108[a]	0.107
5.00	0.0118[a]	
7.00	0.0102[a]	0.074
0.30[b]	0.09	0.35

[a] Values are for the slower phase determined using a biexponential decay model.
[b] Data for scrambled sequence, GGTGGTGGTTGTGGT.

previous observations that chelation of divalent metals by citrate leads to inhibition, suggesting that the nuclease activities in serum and plasma are similar.

The kinetic data in plasma and serum, together with the prothrombin binding data, suggest that the degradation of GS-522 in plasma is inhibited by pre-equilibrium binding to plasma protein(s), presumably prothrombin, and by the presence of citrate. The assumption of a rapid pre-equilibrium binding of GS-522 to prothrombin is justified considering the small K_P value. Assuming a diffusion controlled rate constant ($\sim 2 \times 10^8\ M^{-1}s^{-1}$) for GS-522 binding to prothrombin (18), the magnitude of the k_{off} should be in the range of 4–20 s^{-1}, far larger than k^s_{obs}. The rate expression for the degradation of GS-522 in plasma without citrate is given in equations 1 and 2,

$$-d[GS\text{-}522]_o dt = k^p_{obs}[GS\text{-}522]_o \quad (1)$$

$$k^p_{obs} = k[E]_o \left(\frac{K_p}{(K_p + [P]_f)}\right) \quad (2)$$

where $[GS\text{-}522]_o$ is the total concentration of GS-522 present and $[E]_o$ is the total concentration of nuclease(s), K_P is the prothrombin dissociation constant, and $[P]_f$ is the free prothrombin concentration. In serum the k^s_{obs} expression is simplified to

$$k^s_{obs} = k[E]_o \quad (3)$$

since there is no prothrombin present. At high GS-522 concentrations $[P]_f = 0$ and thus $k^P_{obs} = k^s_{obs}$. At low [GS-522], $[P]_{total} \approx [P]_f$ and assuming a $[P]_{total} = 1$ μM in the monkey and substituting in the K_d value determined above for hPT and GS-522 for K_P, the calculated ratio of k^s_{obs}/k^P_{obs} is ~20. The experimental ratio using the k^P_{obs} and k^s_{obs} values at 0.1 μM GS-522 (k^P_{obs} corrected for citrate inhibition) is 4.5. There are several possible reasons for the discrepancy between the calculated and experimental ratios of k^s_{obs}/k^P_{obs}. The K_P for monkey prothrombin could be higher than for hPT, the monkey prothrombin concentration could be lower than 1 μM, or the divalent metals present in plasma could destabilize the G-quartets and thus increase K_P (19). An overall scheme addressing these possibilities is depicted below,

$$\begin{array}{ll} [GS\text{-}522]_f & P \\ E \downarrow k & \rightleftharpoons [GS\text{-}522]_b \\ \text{Metabolism} & K_p \end{array}$$

where $[GS\text{-}522_b]$ and $[GS\text{-}522_f]$ are prothrombin bound and free GS-522. A large body of data exists on the stability of modified and unmodified oligodeoxynucleotides using isolated nucleases, cellular extracts, and tissue culture media (20, 21, 22). However, limited stability data have been generated in plasma or serum and much of this data suffers from poor quantitation or insufficient kinetic characterization. For an unmodified 21-mer, the half-life in 10% fetal calf serum was reported to be less than 5 min (23). In 100% rat serum, the half-life of a 16-mer was reported to be less than 1 min (24) and in 100% bovine calf serum, Wickstrom reported the complete degradation of a 15-mer in 15 min, suggesting a maximum half-life of 3 min or less (21). In a comparative stability study between various mammalian plasmas, Eder et al. has reported a k_{obs} value of 0.09 min^{-1} for a 15-mer in human plasma (similar to the k^s_{obs} for GS-522 in monkey serum) and a much faster degradation rate for single-stranded versus double-stranded oligodeoxynucleotides (25). The half-lives determined for GS-522 in serum are in the range for those reported allowing for variations in sequence, dilution, and processing of the sera. To determine what, if any, effect the tertiary structure of GS-522 might have on nuclease resistance, the stability of the scrambled sequence was also studied in 90% monkey serum. The scrambled sequence does not inhibit thrombin nor does it appear to have a defined solution structure. The data in Table II show that at 0.3 μM, the scrambled sequence is approximately four-fold more susceptible to degradation than GS-522 in monkey serum. While the increased nuclease(s) resistance of GS-522 is likely due to its solution structure, it is not clear whether the effect represents decreased affinity for the nuclease(s) or increased affinity to putative serum binding proteins relative to the scrambled sequence. The half-life of 1.5 min obtained for the scrambled sequence is at the limit of what can be determined using the kinetic methods employed in this study.

K_M and V_{max}

The kinetic treatment of GS-522 degradation described above assumes that the nuclease(s) is not operating under conditions where the concentration of substrate is approaching saturation. The observed rate constants (k_{obs}) calculated in serum and plasma are composite terms that include the total nuclease(s) concentration ($[E]_o$), k_{cat}, K_M and other putative equilibria (in plasma) such as the binding of GS-522 to prothrombin and citrate chelation of divalent metals. At low GS-522 concentration where $[GS\text{-}522] \ll K_M$ the k term from equation 2 and 3 is equal to:

$$k = \frac{K_{cat}}{K_M} \quad (4)$$

In order to calculate the K_M and V_{max} for the enzymatic degradation of GS-522 in serum, where the effects of pro-

thrombin and citrate are not present ($k^s_{obs} = k[E]_o$), an initial rate study was carried out at varying GS-522 concentrations and a single homogeneous population of nuclease(s) was assumed. The initial reaction rates versus the initial GS-522 concentration are plotted in Figure 3. The K_M and V_{max} values calculated by fitting the curve to the Michaelis-Menton equation are 8.4 μM and 0.87 μM min^{-1}, respectively. Since $V_{max} = k_{cat}[E]_o$, rearranging equation 3 and 4,

$$k^s_{obs} = \frac{V_{max}}{K_M} \quad (5)$$

and substituting the K_M and V_{max} values into equation 5 yields a value of 0.104 min^{-1} for k^s_{obs} which is very similar to the average value of k^s_{obs} (0.090 min^{-1}) determined in monkey serum using the kinetic analysis described above. The K_M value is ~6-fold lower than for the 15-mer oligodeoxynucleotide reported by Eder (25), suggesting that GS-522 has a higher affinity for the nuclease(s), but a lower k_{cat}.

Metabolic Products

The chromatograms obtained after incubating 20 μM GS-522 for 0, 30, 60 and 120 min in monkey plasma are shown in Figure 4. As degradation proceeds, additional peaks with shorter retention times appear in the chromatograms.

The metabolic profile for the scrambled oligodeoxynucleotide shows a similar "ladder-like" degradation pattern (data not shown). The lack of shorter oligodeoxynucleotides at the early time points for GS-522 suggests that the endonuclease activity in plasma is very low relative to the exonuclease activity. This is consistent with many reports in the literature that identify the 3′-exonucleases as the primary enzymes responsible for oligodeoxynucleotide degradation (23, 24) in serum and plasma. The metabolite peaks have been identified as the n − 1 to n − 6 oligodeoxynucleotides by comparison to the retention times of authentic samples of the 5′ truncated oligodeoxynucleotides. The identification is possible even though cleavage occurs from the 3′ end of the oligodeoxynucleotide because the GS-522 molecule has a symmetrical sequence and exonuclease activity results in the sequential removal of single nucleotides from the oligodeoxynucleotide.

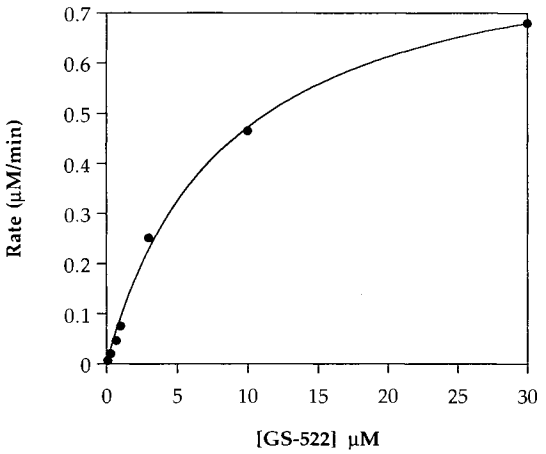

Fig. 3. The effect of [GS-522] on the initial rate of GS-522 degradation in monkey serum. K_M = 8.4 μM; V_{max} = 0.87 μM min^{-1}.

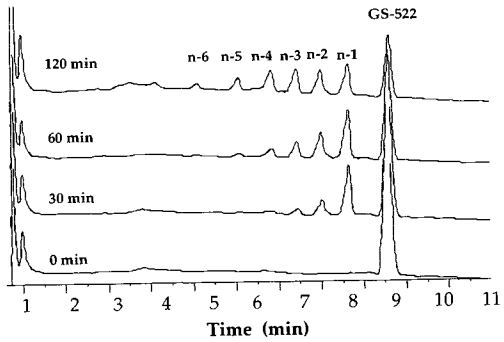

Fig. 4. Chromatograms of sequential degradation of GS-522 in monkey plasma. [GS-522] = 20 μM; T = 37°C.

In summary, we have developed a highly sensitive anion-exchange HPLC method for the determination of a novel oligodeoxynucleotide in plasma and serum. The method is capable of supporting preclinical pharmacokinetic studies in dog and monkey. The greater stability of GS-522 in monkey plasma relative to serum is due to a pre-equilibrium binding to prothrombin and the presence of citrate in plasma. The enhanced stability observed with GS-522 compared to the scrambled sequence is likely a reflection of the solution structure of the former. The half-lives observed for GS-522 in the various plasmas are all greater than the observed *in vivo* elimination half-life reported in monkeys (4), suggesting that *in vivo* plasma metabolism plays a minor role in the overall *in vivo* elimination. Therefore, synthetic strategies designed to decrease the enzymatic lability of an oligodeoxynucleotide will not necessarily increase the *in vivo* plasma half-life. Finally, analysis of the metabolites confirms that the predominant degradation pathway in plasma is the result of exonuclease activity.

ACKNOWLEDGMENTS

The authors would like to thank Lou Bock, Steve Coutre and Linda Griffin for many helpful discussions and Kaye Donnelly for her careful preparation of this manuscript.

REFERENCES

1. J. F. Milligan, M. D. Matteucci, and J. C. Martin. Current concepts in antisense drug design. *J. Med. Chem.* 36:1923–1937 (1993).
2. S. L. Young, S. H. Krawczyk, M. D. Matteucci, and J. J. Toole. Triple helix formation inhibits transcription elongation *in vitro*. *Proc. Natl. Acad. Sci. USA* 88:10023–10026 (1991).
3. L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas, and J. J. Toole. Selection of single-stranded DNA melocules that bind and inhibit human thrombin. *Nature* 355:564–566 (1992).
4. L. C. Griffin, G. F. Tidmarsh, L. C. Bock, J. J. Toole, and L. K. Leung. *In vivo* anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracorporeal circuits. *Blood* 81:3271–3276 (1993).
5. K. Y. Wang, S. McCurdy, R. G. Shea, S. Swaminathan, and P. H. Bolton. A DNA aptamer which binds to and inhibits thrombin exhibits a new structural motif for DNA. *Biochemistry* 32:1899–1904 (1993).
6. R. F. Macaya, P. Schultze, F. W. Smith, J. A. Roe, and J.

Feigon. Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc. Natl. Acad. Sci. USA* 90:3745–3749 (1993).
7. L. R. Paborsky, S. N. McCurdy, L. C. Griffin, J. J. Toole, and L. K. Leung. The single-stranded DNA aptamer-binding site of human thrombin. *J. Biol. Chem.* 268:20808–20811 (1993).
8. E. W. Salzman. Low-Molecular-Weight Heparin and Other New Antithrombotic Drugs. *N. Engl. J. Med.* 326:1017–1019 (1992).
9. L. G. Davis, M. D. Dibner, and J. F. Battey. Basic methods in molecular biology. Elsevier, Amsterdam, 44–46 (1986).
10. PCNONLIN® Version 4.2, Software for the Statistical Analysis of Nonlinear Models on Micros, Statistical Consultants, Inc., Lexington, KY.
11. H. Akaike. A Bayesian analysis of the minimum AIC procedure. *Ann Inst. Statist. Math.* 30:9–14 (1978).
12. W. Haupt and A. Pingoud. Comparison of several high-performance liquid chromatography techniques for the separation of oligodeoxynucleotides according to their chain lengths. *J. Chromatogr.* 260:419–427 (1983).
13. J. M. Stephens, L.-C. Yuan, and W. A. Lee. Extinction Coefficient Determination by Quantitative Base Composition Analysis of GS-522, a Novel Oligodeoxynucleotide Inhibitor of Thrombin. Abstract No. *13th International Symposium on the HPLC Analysis of Proteins, Peptides, and Polynucleotides,* San Francisco, CA, December 1993.
14. J. H. Jandl. Blood, Textbook of Hematology. Little, Brown and Co., Boston, 1987.
15. Q. Wu, M. Tsiang, and J. E. Sadler. Localization of the single-stranded DNA binding site in the thrombin anion-binding exosite. *J. Biol. Chem.* 267:24408–24412 (1992).
16. P. J. Braun and K. M. Szewczyk. Relationship between total prothrombin, native prothrombin and the International Normalized Ratio (INR). *Thrombosis and Haemostasis* 68:160–164 (1992).
17. N. B. Kurnick. Desoxyribonuclease activity of sera of man and some other species. *Arch Biochem Biophys.* 43:97–107 (1953).
18. D. Riesner, A. Pingoud, D. Boehme, F. Peters, and G. Maas. Distinct Steps in the Specific Binding of tRNA to Aminoacyl-tRNA Synthetase. *Eur. J. Biochem.* 68:71–80 (1976).
19. R. F. Macaya, P. Shultze, F. W. Smith, J. A. Roe, and J. Feigon. Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc. Nat'l. Acad. Sci. USA.* 90:3745–3749 (1993).
20. C. A. Stein, C. Subasinghe, K. Shinozuka, and J. Cohen. Physiochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acid Res.* 16:3209–3221 (1988).
21. E. Wickstrom. Oligodeoxynucleotide stability in subcellular extracts and culture media. *J. Biochem. Biophys. Methds.* 13:97–103 (1986).
22. S. Akhtar and R. L. Juliano. Stability of antisense DNA oligodeoxynucleotide analogs in cellular extracts and sera. *Life Sciences* 49:1793–1801 (1991).
23. J.-P. Shaw, K. Kent, J. Bird, J. Fishback, and B. Froehler. Modified deoxyoligodeoxynucleotides stable to exonuclease degradation in serum. *Nuc. Acids Res.* 19:747–750 (1991).
24. P. C. de Smidt, T. L. Doan, S. de Falco, and T. J. C. van Berkel. Association of antisense oligodeoxynucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nuc. Acids Res.* 19:4695–4700 (1991).
25. P. S. Eder, R. J. DeVine, J. M. Dagle and J. A. Walder. Substrate specificity and kinetics of degradation of antisense oligodeoxynucleotides by a 3′-exonuclease in plasma. *Antisense Res. and Dev.* 1:141–151 (1991).