0026-895X/94/050932-12$03.00/0

MOLECULAR PHARMACOLOGY, 45:932-943

Biodistribution and Metabolism of Internally ^{3}H-Labeled Oligonucleotides. I. Comparison of a Phosphodiester and a Phosphorothioate

HOWARD SANDS,[1] LORRAINE J. GOREY-FERET, ANTHONY J. COCUZZA, FRANK W. HOBBS, DENNIS CHIDESTER, and GEORGE L. TRAINOR

DuPont Merck Pharmaceutical Company, Wilmington, Delaware 19880

Received November 4, 1993; Accepted February 10, 1994

SUMMARY

Biodistribution and metabolism of oligonucleotides were determined using a ^{3}H-labeled 20-nucleotide phosphodiester and its phosphorothioate analog. The oligonucleotides were radiolabeled by ^{3}H-methylation of an internal deoxyctidine with *Hha*I methylase and *S*- [^{3}H]adenosylmethionine. Biodistribution studies were conducted after intravenous injection of 6 mg/kg (5 μCi) oligonucleotide. Metabolism of the oligonucleotides was determined by paired-ion high performance liquid chromatography. After phosphodiester injections, radiolabel rapidly cleared the blood. Relative initial concentrations were as follows: kidney > blood > heart > liver > lung > spleen. Radiolabel in spleen peaked at 1 hr and remained elevated for 24 hr. At 2 hr the concentration in all organs, except spleen, was equal to that in blood. High performance liquid chromatographic analysis of the kidney, liver, and spleen extracts and urine indicated extremely rapid metabolism to monomer. Results of studies after the injection of phosphorothioate oligonucleotide differed from those using the phosphodiester. Despite its rapid clearance from blood, phosphorothioate accumulated rapidly in all tissues, especially the kidney. Kidney uptake increased over time, remaining very high for 24 hr. Ratios of organ to blood concentrations at 2 hr for all organs were 5:1 or greater. Kidney and liver ratios were 84:1 and 20:1, respectively. Analysis of the kidney and liver extracts and urine indicated that slow metabolism occurred. These data suggest that phosphodiester oligonucleotides would have limited therapeutic utility. The stability and organ distribution of the phosphorothioate oligonucleotide imply that such oligonucleotides may have therapeutic potential.

Antisense oligonucleotides designed to block the synthesis of specific disease-related proteins have been hailed as the next generation of drugs (1–3). The antisense concept has great appeal and exhibited efficacy *in vitro* (4), but many issues critical to drug development remain unresolved. For example, rapid *in vitro* degradation of the phosphodiester oligonucleotides by exo- and endonucleases (4) has lead to the search for more enzyme resistant and stable molecules. Substitution of a sulfur atom or methyl group for an oxygen atom in the phosphodiester linkages of conventional oligonucleotides generates phosphorothioate and methylphosphonate oligonucleotides, respectively, analogs that have much greater *in vitro* stability (5). Although several reports have dealt with the biodistribution of various types of radiolabeled nucleotides (6–10), no systematic comparison of oligonucleotides analogs has been reported. Because oligonucleotides can be degraded by a variety of enzymes (and reincorporated into macromolecules), the site and nature of the radiolabel potentially can have a profound influence on the observed biodistribution and metabolism.

Several reports on the biodistribution and metabolism of oligonucleotides have used oligonucleotides radiolabeled at a problematic site, namely one end of the oligonucleotide. The conventional way to label an oligonucleotide is to use T4 polynucleotide kinase to transfer a [^{32}P]phosphate group from ATP to the 5′ end of the oligonucleotide. This radiolabeling strategy was used in the biodistribution studies of de Smidt *et al.* (6), Goodarzi *et al.* (7), and Inagaki *et al.* (8). Similarly, Boutorine *et al.* (11) recommended 5′ enzymatic tagging with [^{32}P]phosphate or [^{35}S]thiophosphate groups. Although 5′-phosphate groups are removed relatively slowly by the enzymatic activities present in the media used for cell culture experiments (frequently containing dilute, heat-inactivated, fetal calf serum), the radiolabel could be removed more rapidly *in vivo*. Whitesell *et al.* (12) used oligonucleotides enzymatically labeled at the 5′ end with [^{32}P]phosphate or at the 3′ end with ^{32}P-nucleotide using dideoxynucleotidyl terminal transferase.

[1] Present address: Lexin Pharmaceutical Co., 111 Rock Road, Horsham, PA 19044.

ABBREVIATIONS: HPLC, high performance liquid chromatography; ID, injected dose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GIT, 4 M guanidine isothiocyanate, 25 mM sodium citrate, and 0.1 M β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; SAM, S-adenosyl-L-[*methyl*-^{3}H]methionine.

Given numerous reports that the major pathway for degradation of oligonucleotides in serum involves 3′-exonucleases, a 3′-labeled nucleotide is least likely to accurately report on the metabolism and biodistribution of the bulk of the oligonucleotide. Finally, Chem *et al.* (9) chemically linked 5-[^{3}H]thymidine to the 5′ end of a methylphosphonate oligonucleotide by means of a phosphodiester linkage. In this case, the linkage between the bulk of the oligonucleotide and the radiolabel is clearly the most labile linkage in the molecule.

In contrast to the aforementioned end-labeling methods, internal radiolabeling should provide more reliable results. Agrawal *et al.* (10) prepared oligonucleotides with all phosphorothioate linkages, which were uniformly labeled at all linkages with ^{35}S, but this radiolabeling strategy cannot be used with other analogs. A single ^{32}P label was incorporated by Zendegui *et al.* (13) (by means of a kinase and a ligase) into a 3′-blocked 38-base oligonucleotide with all phosphodiester linkages. This radiolabeling strategy has the same theoretical advantage as the strategy used in our work (but it should be noted that the metabolism of the aforementioned oligonucleotide was studied by 5′-labeling nonradioactive oligonucleotide recovered from tissue samples).

Using the aforementioned differing radiolabeling strategies and differing analytical methodologies, a variety of conclusions about the biodistribution of unmodified and modified oligonucleotides have been reported. de Smidt *et al.* (6) studied the biodistribution in rats of a ^{32}P-end-labeled phosphodiester oligonucleotide, as a control for a cholesterol-oligonucleotide complex. ^{32}P-Oligonucleotide cleared rapidly from the blood, accumulated, and was degraded in the kidney and excreted in the urine. A study using a ^{32}P-end-radiolabeled phosphorothioate oligonucleotide was reported by Goodarzi *et al.* (7). Oligonucleotide accumulated in the liver of mice, where it was degraded. Similar results, i.e., rapid liver uptake and degradation, have been reported by Inagaki *et al.* (8). The biodistribution and degradation of methylphosphonates radiolabeled at position 2 with a [^{3}H]methyl group were studied in mice by Chem *et al.* (9). Rapid plasma clearance of the ^{3}H and low levels of accumulation were seen in most organs. Degradation of the 12-mer to the 11-mer occurred in kidney, lung, and spleen but not in plasma.

More meaningful data can be generated by the study of uniformly labeled oligonucleotides. In contrast to the results reported with the use of a ^{32}P-end-radiolabeled phosphorothioate oligonucleotide, the biodistribution and metabolism studies by Agrawal *et al.* (10) used uniformly ^{35}S-labeled phosphorothioate oligonucleotides. Those authors detected the slow degradation of oligonucleotide in the liver of mice and also found rapid blood clearance, high levels in the kidneys, and excretion through the urine. In addition to degradation, they found chain extension in the liver. Degradation products were seen in the liver and urine.

For antisense technology to succeed, a thorough understanding of the physiology and metabolism of these macromolecules is essential. It is difficult to make comparisons and draw conclusions from the studies cited above because of the use of different labeling methods, sizes of oligonucleotides, backbone modifications, and animal species. The placement of the radiolabel within the oligonucleotide has dramatic effects on the observed biodistribution and metabolism. We have completed a direct comparison, under identical conditions (using a novel radiolabeling procedure and paired-ion chromatography), of the biodistribution and metabolism in mice of a phosphodiester and a phosphorothioate oligonucleotide (of the same length and sequence) with an internal radiolabel (^{3}H) carried at the same position. Because the sequence was chosen based on the specificity of the radiolabeling technology, these oligonucleotides are not directed at a specific mouse sequence or function. The data generated suggest that the phosphodiester oligonucleotide would have limited therapeutic utility due to its low organ accretion and rapid metabolism. In contrast, the stability and organ distribution of the phosphorothioate oligonucleotide imply that oligonucleotides bearing this modification may have therapeutic potential.

Materials and Methods

Preparation of Oligonucleotides

Synthesis. Phosphodiester oligodeoxyribonucleotides were prepared on the 10-μmol scale using commercially available phosphoramidites and a model 394 DNA synthesizer (Applied Biosystems, Forest City, CA), following the manufacturer's instructions. Phosphorothioate oligodeoxyribonucleotides were prepared in a similar manner, using tetraethylthiuram disulfide as the sulfurizing reagent. After cleavage and deprotection with concentrated ammonia (4–16 hr, 55°), crude 5′-dimethoxytritylated oligonucleotides were purified by nondenaturing HPLC (14) and desalted on NAP-10 columns (Bio-Rad, Rockville Center, NY). The oligonucleotide was converted to its sodium salt by passing the solution of the resulting triethylammonium salt in distilled water through a ~1-g column of Dowex-50 (sodium form). Solutions of both types of oligonucleotides in distilled water were quantitated by UV absorbance, using extinction coefficients estimated after accounting for nearest-neighbor interactions (171,000 liters/mol/cm for the 20-nucleotide oligonucleotide and 97,000 liters/mol/cm for the 10-nucleotide oligonucleotide) (15).

^{3}H-Labeling of the 20-nucleotide phosphodiester oligodeoxyribonucleotide. The 20-nucleotide phosphodiester to be labeled (5′-TCATGCTCATGCGCTCATGC-3′) (27.5 nmol), a complementary 10-nucleotide oligonucleotide [5′-TGAGC(Me)GCATG-3′] (27.5 nmol), Tris·HCl (125 mM, pH 7.5), EDTA (25 mM, pH 7.5), and β-mercaptoethanol (12.5 mM) in 100 μl of solution were heated to 95° and allowed to cool. Six aliquots of [*methyl*-^{3}H]SAM (totaling 1 mCi, 13.6 nmol; DuPont, Boston, MA), in aqueous sulfuric acid, pH 2, were lyophilized, neutralized, combined with 150 μl of aqueous HEPES sodium salt (0.15 M), and transferred to the duplex stock. *Hha*I methylase (25 μl of 125 units/μl; New England Biolabs, Beverly, MA) was added and the reaction was incubated at 37° for 3 hr. After heating at 95° for 10 min, nucleic acids were isolated by ethanol precipitation (1/5 volume of 5 M NaCl and 2 volumes of ethanol). The desired 20-nucleotide oligonucleotide was separated from the 10-nucleotide template by preparative denaturing PAGE with detection by UV shadowing. The tritiated 20-nucleotide oligonucleotide was isolated from the gel by repeated soaking with ammonium acetate (0.3 M), lyophilization, and ethanol precipitation. The final product contained 442 μCi (6.0 nmol) of tritiated methyl group on the underlined cytosine residue (31.8 μCi/nmol) and 13.9 nmol of oligonucleotide (a yield of 51%).

^{3}H-Labeling of the 20-nucleotide phosphorothioate oligodeoxyribonucleotide. The 20-nucleotide phosphorothioate to be labeled (5′-TCATGCTCATGCGCTCATGC-3′) (55 nmol), a complementary phosphodiester 10-nucleotide oligonucleotide (5′-TGAGC(Me)GCATG-3′) (55 nmol), Tris·HCl (125 mM, pH 7.5), EDTA (25 mM, pH 7.5), and β-mercaptoethanol (12.5 mM) in 100 μl of solution were heated to 95° and allowed to cool. Six aliquots of [*methyl*-^{3}H]SAM (totaling 1 mCi = 13.6 nmol; DuPont), in aqueous sulfuric acid, pH 2, were lyophilized, neutralized, combined with 150 μl of aqueous HEPES sodium salt (0.15 M), and transferred to the duplex stock. *Hha*I methylase (50 μl of 125 units/μl; New England Biolabs)

was added and the reaction was incubated at 37° for 18 hr. After heating at 95° for 10 min, nucleic acids were isolated by desalting on a NAP-25 column (Bio-Rad). The desired 20-nucleotide oligonucleotide was separated from the 10-nucleotide template by preparative denaturing PAGE with detection by UV shadowing. The tritiated 20-nucleotide oligonucleotide was isolated from the gel by repeated soaking with ammonium acetate (0.3 M). The oligonucleotide was isolated from the soak solutions by absorbing the solutions onto a SEP-PAK (Waters, Milford, MA) equilibrated with aqueous ammonium acetate (50 mM), washing with 10 ml of aqueous ammonium acetate, elution with 3 ml of 1:1 methanol/aqueous ammonium acetate, and lyophilization. The final product contained 235 μCi (3.2 nmol) of tritiated methyl group on the underlined cytosine residue (10.8 μCi/nmol) and 21.8 nmol of oligonucleotide (a yield of 40%).

Mice

Either radiolabeled oligonucleotide or inulin was injected at a dose of 6 mg/kg (approximately 5 μCi), via the tail vein, into male CF1 mice weighing approximately 28 g. Animals were sacrificed by CO_2 asphyxiation at the indicated times. A minimum of five mice were used at each time point. Blood was drawn from the heart and the indicated organs were removed for determination of radioactivity. Twenty-four-hour urine samples were collected in metabolic cages, frozen, stored at −4°, centrifuged through 0.2-μm filters, diluted appropriately in phosphate buffer, and analyzed using the paired-ion chromatography protocol described below.

Biodistribution

Whole organs were weighed, and then approximately 100 mg of the tissue were added to glass scintillation vials containing 1 ml of tissue solubilizer (Beckman, Fullerton, CA). Samples were digested at 55° overnight. Decolorization of samples was accomplished by adding 100 μl of H_2O_2. After incubation for an additional 30 min at 55°, chemiluminescence was reduced by the addition of 50 μl of glacial acetic acid. ReadyOrganic liquid scintillation cocktail (Beckman) was added to all samples, which were then counted with a Beckman 5000 CE liquid scintillation counter. All counts were quench corrected using the Beckman H# method and are reported as dpm. Data were calculated as both the percentage of ID/organ and percentage of ID/g and are reported as the mean ± standard error of the percentage of ID/g of five or more determinations. For the percentage of ID/organ calculation the following assumptions were made: total blood volume was 5% of body weight and total muscle content was 45% of body weight.

Tissue Extractions

Upon removal, the major metabolic organs (kidney, liver, and spleen) were frozen on dry ice and kept at −70 ° until oligonucleotide extractions were performed. Approximately 200 mg of tissue were placed in a microfuge tube containing 500 μl of GIT, to terminate metabolism and denature proteins. The sample was sonicated for approximately 1 min with a miniature ultrasonic probe (Conoco Specialty Instruments, Wilmington, DE). The GIT lysate was diluted with 2.5 ml of water, and 2.5 ml of phenol/chloroform (1:1, v/v) were added. The sample was vortexed and centrifuged at 10,000 × *g* for 30 min. The aqueous layer was carefully removed and the remaining organic layer was re-extracted with an additional 2.5 ml of water. Aqueous layers from the two extractions were pooled, dried down, and resuspended in 1 ml of water. In some cases, to increase the number of counts available for HPLC analysis, three samples of tissue were extracted and combined. Preliminary experiments indicated that >70% of the radioactive nucleic acids in the tissue were recovered and observed by HPLC.

HPLC Analysis

The radioactive nucleic acids found in the tissue extracts were analyzed by paired-ion chromatography on a Microsorb MV-C4 column (Rainin Instruments, Woburn, MA), using an Isco (Lincoln, NE) pump and gradient maker. Buffer A was 5 mM PIC-A reagent (Millipore Corp., Bedford, MA) in water and buffer B was 4:1 acetonitrile/water. The following gradient was used, with a flow rate of 1 ml/min: (a) 0–5 min, 0% buffer B; (b) 5–20 min, 0–35% buffer B; (c) 20–80 min, 35–65% buffer B. (The column was equilibrated with 100% buffer A for 12 min between runs.) The eluate from the column (1 ml/min) was mixed with ReadyFlow scintillation cocktail (Beckman) (delivered at a flow rate of 4 ml/min) and analyzed using a Betram on-line β-radiation detector (IN/US Systems, Inc., Fairfield, NJ) with a counting efficiency of 50%.

Serum Stability Studies

Approximately 1 μCi of ^{3}H-labeled oligonucleotide was added to 2 ml containing 1 μM oligonucleotide and incubated in 100% non-heat-inactivated mouse serum (Sigma Chemical Co., St. Louis, MO) at 37°. At the indicated time one tenth of the incubation solution was removed and added to 500 μl of GIT. Extraction of the ^{3}H-labeled oligonucleotides and metabolites was carried out according to the procedure described above.

Autoradiography

Mice were injected and sacrificed at designated times as described above. The kidneys were removed, flash frozen on dry ice, and stored at −70°. Frozen kidneys were cut into 15-μm slices on a cryostat and placed on glass slides. The slices were dried and fixed in a desiccator with powdered paraformaldehyde. After being defatted by a series of washes with increasing alcohol concentrations, the slides were placed on ^{3}H-Hyperfilm (RPN12; Amersham, Arlington Heights, IL) and exposed for 8 weeks. The film was then developed by standard procedures.

Serum Albumin Binding

To assess the ability of the oligonucleotides to bind to serum albumin, each drug or oligonucleotide (10^{-5} M total concentration, containing approximately 1 μCi of radiolabel) was added to 1 ml of 0.42% (w/v) human serum albumin (Sigma) in the upper chamber of an UltraFree-Cl filter unit with a 30,000 nominal molecular weight cut-off (Millipore). A 50-μl aliquot was taken for liquid scintillation counting. After incubation for 30 min at room temperature, the filter unit was centrifuged at 3000 rpm for 10 min and a 50-μl aliquot of the filtrate in the lower chamber was counted. Nonspecific binding was determined by parallel determinations in tubes lacking the addition of serum albumin. Specific binding was determined by subtracting the nonspecific binding value from the albumin binding value. All assays were done in duplicate. [^{3}H]Digitoxin and [^{3}H]digoxin, obtained from DuPont, were used as standards for comparison. Nonradiolabeled digitoxin and digoxin were obtained from Sigma.

Results

Radiolabeling. Oligonucleotides for biodistribution studies were labeled with *Hha*I methylase by the method outlined in Fig. 1. To be substrates for *Hha*I methylase, the 20-nucleotide oligonucleotides were hybridized to a complementary 10-nucleotide phosphodiester. To prevent the complement from also being labeled, this 10-nucleotide oligonucleotide was synthesized with a methylated GCGC site. Using *Hha*I methylase and the conditions described above, roughly 80% of the tritium in 1 mCi of [^{3}H]SAM was transferred to the all-phosphodiester duplex. When the strand to be labeled contained all phosphorothioate linkages, the mixed-backbone duplex was a somewhat poorer substrate. Modified methylation conditions, however, transferred more than half of the radioactivity to the phosphorothioate strand. Most of the unincorporated [^{3}H]SAM was removed by ethanol precipitation or gel filtration (the phosphorothioate strand did not precipitate well) and then the labeled 20-nucleotide oligonucleotide was separated from the

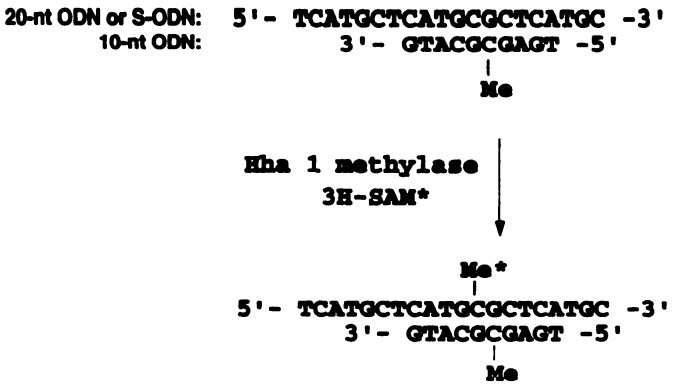

Fig. 1. Scheme for radiolabeling oligonucleotides. A tritiated methyl (*Me*) group was enzymatically attached to the 5-position of the indicated deoxycytidine in the 20-nucleotide phosphodiester (*ODN*) or phosphorothioate (*S-ODN*). The complementary 10-nucleotide oligonucleotide was synthesized with a 5-methyldeoxycytidine residue to block tritiation of this strand. *nt*, nucleotide.

10-nucleotide template by preparative PAGE. The overall purification process returned about half of the input oligonucleotide (by UV quantitation) and therefore several hundred microcuries of the tritiated oligonucleotide. The specific activity of the oligonucleotides ranged from 10 to 30 μCi/nmol. As expected, the purified labeled oligonucleotides were homogeneous when analyzed by PAGE (with detection by fluorography) and by several HPLC systems (see Figs. 3a and 4a; other data not shown). Experiments with *Hha*I endonuclease demonstrated that incorporation of a single tritiated methyl group prevented cutting and that all of the tritium label was incorporated at the GCGC recognition site (data not shown).

HPLC analysis and serum stability. The resolution capabilities of the paired-ion chromatography method developed for the study of tissue metabolism and serum stability of radiolabeled oligonucleotides can be seen in Fig. 2. Fig. 2a shows the separation seen with the two possible fragments formed after cutting the 20-nucleotide phosphodiester on either side of the methylated deoxycytidine. Fig. 2b illustrates the resolution seen with 15-, 18-, and 20-nucleotide phosphorothioate oligonucleotides. The elution times of the ultimate monomer metabolites and of the radiolabeled 20-nucleotide phosphodiester are shown in Fig. 2c. Clearly this method was able to resolve the starting material from the expected metabolic products produced by the removal of two or more bases, as well as from terminal monomers. In all of the studies described above the elution times for the 20-mers were approximately 70 min.

Incubation of the ^{3}H-phosphodiester oligonucleotide (retention time, 72 min) with undiluted mouse serum eventually produced two major radiolabeled products (Fig. 3). Coinjection with a variety of nucleic acid standards established that these products were 5-[^{3}H]methyl-2′-deoxycytidine (5 min) and [^{3}H]thymidine (7 min). Further incubation resulted in increased conversion of 5′-methyl-2′-deoxydeoxyctidine to thymidine. Neither of these metabolites was present at the same time as intact oligonucleotide, so degradation was not highly processive. The apparent half-life of the intact oligonucleotide was about 30 min. From about 0.5 to 2 hr, a significant population of intermediate-sized oligonucleotides was observed that shortened with continued exposure to serum. Labeled mononucleotide(s) began to be released from these intermediates

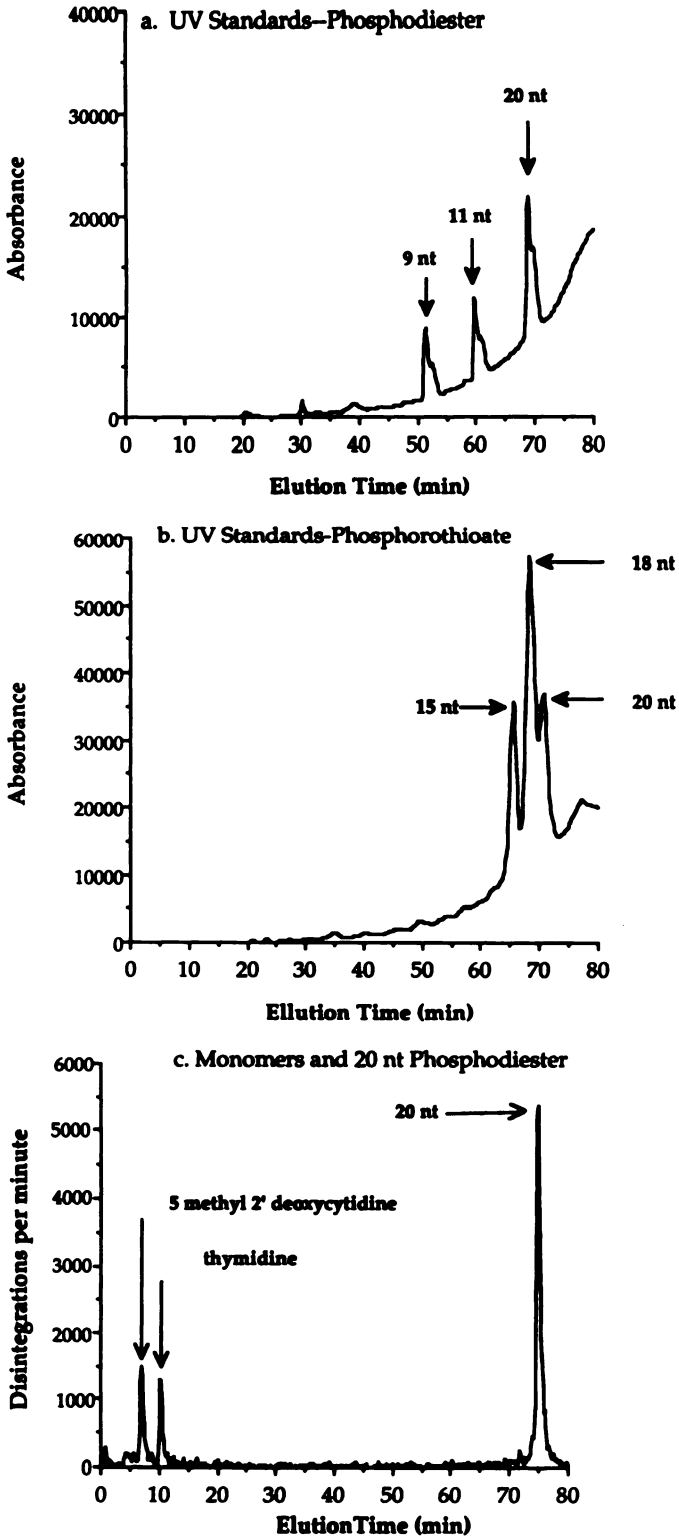

Fig. 2. Paired-ion chromatography of oligonucleotides and their metabolites. a, UV phosphodiester markers: b, UV phosphorothioate markers; c, ^{3}H-labeled 20-nucleotide phosphodiester oligonucleotide and ^{3}H-metabolites.

when the average degradation product was about 10 bases in size. This pattern is consistent with previous reports stating that 3′-exonucleases are predominantly responsible for oligonucleotide degradation in serum.

The HPLC elution profile of the ^{3}H-phosphorothioate oli-

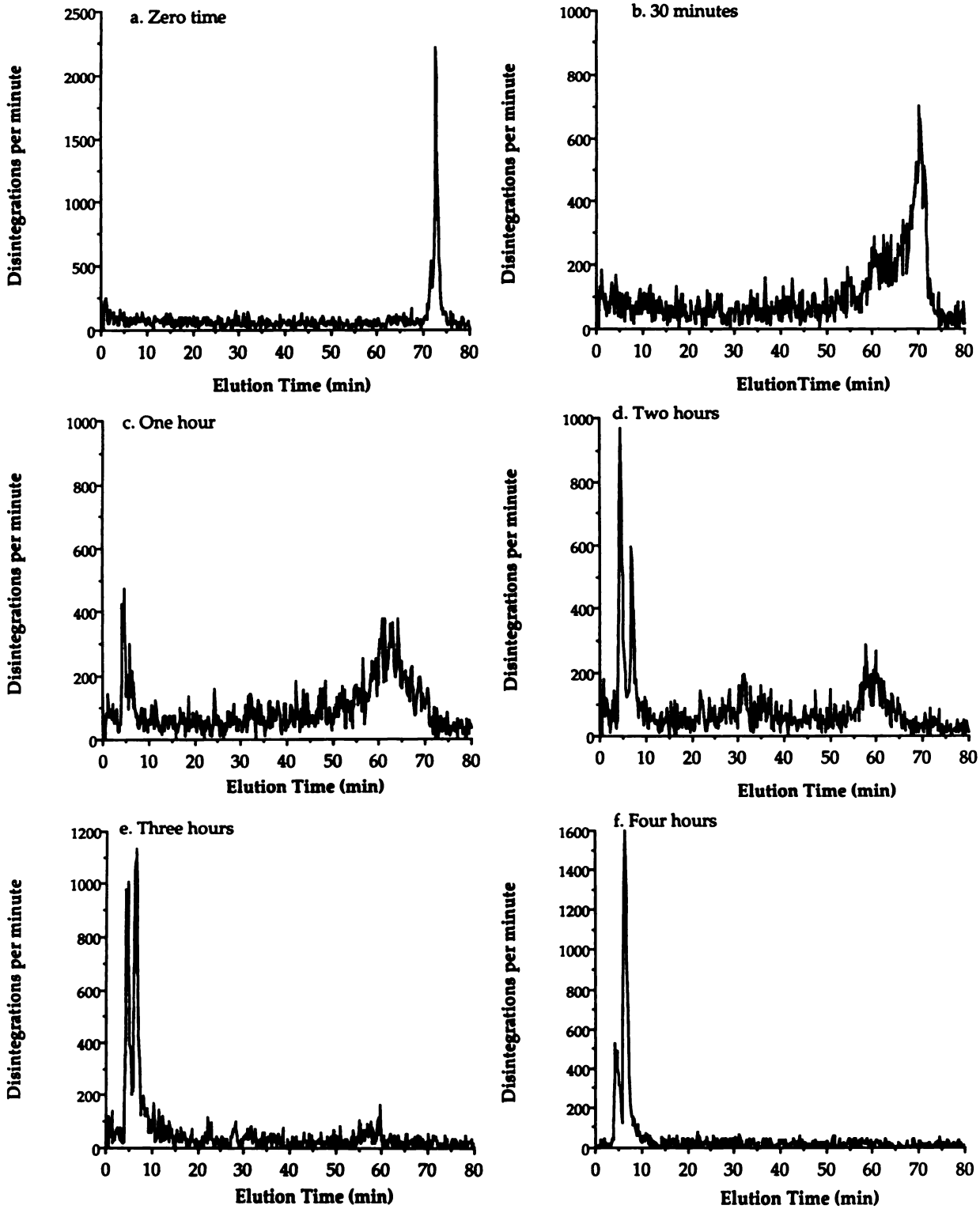

Fig. 3. Paired-ion chromatographic analysis of mouse serum incubated with 1 μM ^{3}H-phosphodiester oligonucleotide for the times indicated.

gonucleotide incubated in mouse serum remained unchanged for up to 5 hr (Fig. 4, a-c), indicating the lack of metabolism. After a 24-hr incubation, a slight broadening of the peak suggested that a minor degree of metabolism occurred via removal of one to three bases (Fig. 4d). No evidence could be found for complete metabolism (i.e., presence of monomer), as represented by the appearance of either the 5- or 7-min peak.

Biodistribution. The biodistribution data generated after intravenous injection of either of the two tritiated oligonucleotides are shown in Tables 1 and 2 and in Fig. 5. Tritium from both oligonucleotides cleared rapidly from the blood, with the phosphorothioate oligonucleotide clearing slightly faster than the phosphodiester oligonucleotide. Twenty-four hours after injection, <0.4% of ID/g for the phosphodiester oligonucleotide

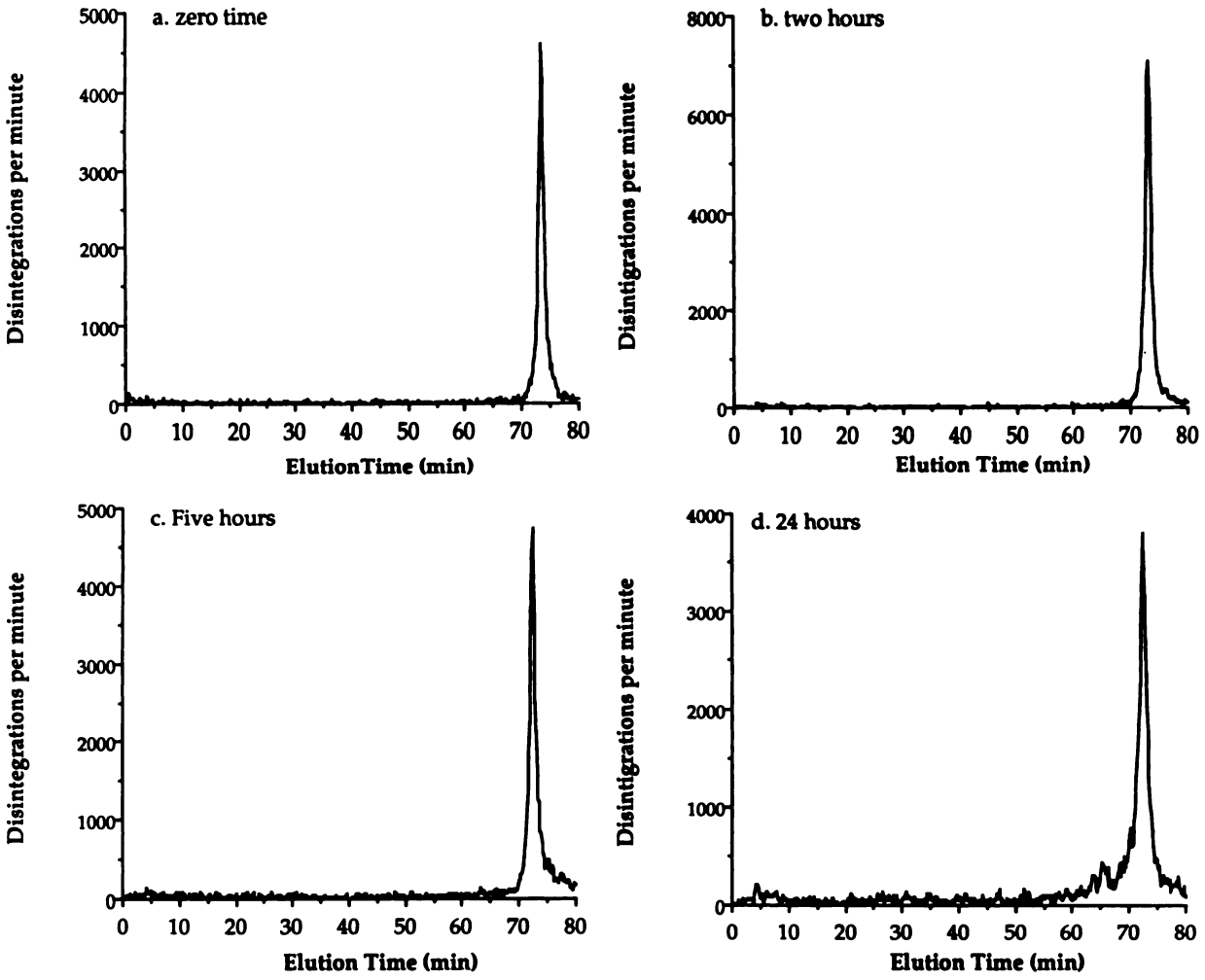

Fig. 4. Paired-ion chromatographic analysis of mouse serum incubated with 1 μM ^{3}H-phosphorothioate oligonucleotide for the times indicated.

TABLE 1

Biodistribution of ^{3}H-phosphodiester oligonucleotide

Values are mean ± standard error (five mice).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
	% of ID/g						
Blood	18.05 ± 0.45	2.06 ± 0.29	1.84 ± 0.15	1.67 ± 0.19	0.78 ± 0.11	1.04 ± 0.09	0.36 ± 0.05
Heart	4.87 ± 0.20	2.13 ± 0.27	1.88 ± 0.17	1.69 ± 0.14	0.84 ± 0.08	1.02 ± 0.08	0.32 ± 0.04
Lung	6.98 ± 0.34	1.90 ± 0.26	1.76 ± 0.13	1.67 ± 0.17	0.76 ± 0.06	1.09 ± 0.10	0.42 ± 0.05
Liver	4.55 ± 0.35	2.45 ± 0.33	2.43 ± 0.16	2.66 ± 0.29	1.18 ± 0.12	1.26 ± 0.10	0.52 ± 0.05
Kidney	13.32 ± 1.96	6.61 ± 0.64	7.91 ± 1.42	5.20 ± 0.15	2.18 ± 0.21	1.43 ± 0.06	0.56 ± 0.07
Muscle	0.67 ± 0.08	1.63 ± 0.25	1.57 ± 0.17	1.58 ± 0.15	0.55 ± 0.06	0.85 ± 0.07	0.26 ± 0.05
Spleen	1.66 ± 0.19	2.59 ± 0.30	3.05 ± 0.16	7.59 ± 0.16	8.04 ± 0.38	8.98 ± 0.85	6.34 ± 0.87
Amount recovered[a]	49.06 ± 3.20	32.60 ± 4.27	31.29 ± 3.18	32.01 ± 3.27	12.67 ± 1.31	15.61 ± 1.08	5.97 ± 0.97

[a] Calculated from percentage of ID/organ values.

could be found in the blood (Fig. 5a). Tritium from the phosphodiester oligonucleotide cleared rapidly from most tissues but accumulated rapidly in spleen. Splenic accretion reached a maximum of 8.98% of ID/g 2 hr after injection. The percentage of ID recovered was calculated by adding the percentage of ID/organ values and is shown in Table 1. The total recovery 1 min after injection (as calculated using the assumptions given in Materials and Methods) was approximately 49% of the ID. The total amount of ^{3}H from the phosphodiester oligonucleotide found in the mice decreased sharply with time, until only 6% of the ID (or 12% of the 1-min recovered doses) could be found in the animal at 24 hr. The large and rapid splenic accumulation seen after the injection of the phosphodiester oligonucleotide was probably due to the accretion by the spleen of terminal (mononucleoside) metabolites of the phosphodiester. After intravenous injection, [^{3}H]thymidine and 5-methyl-2′-[^{3}H]deoxycytidine were rapidly accumulated by the spleen (13.25 ± 1.93 and 7.20 ± 0.90% of ID/g, respectively, at 1 hr). Estimates of volume of distribution and blood half-life for whole-body elimination of the phosphodiester oligonucleotide are 80.7 ml

TABLE 2

Biodistribution of ^{3}H-phosphorothioate oligonucleotide

Values are mean ± standard error (five mice).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
	% of ID/g						
Blood	26.41 ± 2.18	8.24 ± 1.03	6.04 ± 0.91	2.59 ± 0.30	0.59 ± 0.12	0.29 ± 0.03	0.31 ± 0.02
Heart	6.08 ± 0.51	2.64 ± 0.45	3.29 ± 0.37	2.60 ± 0.24	2.01 ± 0.36	1.73 ± 0.30	1.11 ± 0.05
Lung	9.01 ± 1.18	3.29 ± 0.48	3.86 ± 0.91	2.39 ± 0.20	2.56 ± 0.58	1.78 ± 0.43	0.79 ± 0.08
Liver	7.19 ± 0.58	4.55 ± 0.44	4.99 ± 0.48	4.79 ± 0.65	5.08 ± 0.58	5.33 ± 0.63	4.07 ± 0.27
Kidney	12.31 ± 0.99	11.17 ± 1.78	16.34 ± 0.66	18.44 ± 1.08	22.15 ± 1.99	22.03 ± 3.38	21.10 ± 1.19
Muscle	0.97 ± 0.16	0.81 ± 0.19	1.48 ± 0.24	1.94 ± 0.20	1.84 ± 0.38	1.78 ± 0.44	1.12 ± 0.09
Spleen	3.19 ± 0.69	3.97 ± 0.50	6.30 ± 0.75	4.52 ± 0.32	4.59 ± 0.83	3.13 ± 0.45	4.19 ± 0.20
Amount recovered[a]	61.87 ± 5.01	47.77 ± 3.56	53.54 ± 5.79	47.95 ± 4.45	51.17 ± 5.93	37.68 ± 2.87	35.02 ± 2.11

[a] Calculated from percentage of ID/organ values.

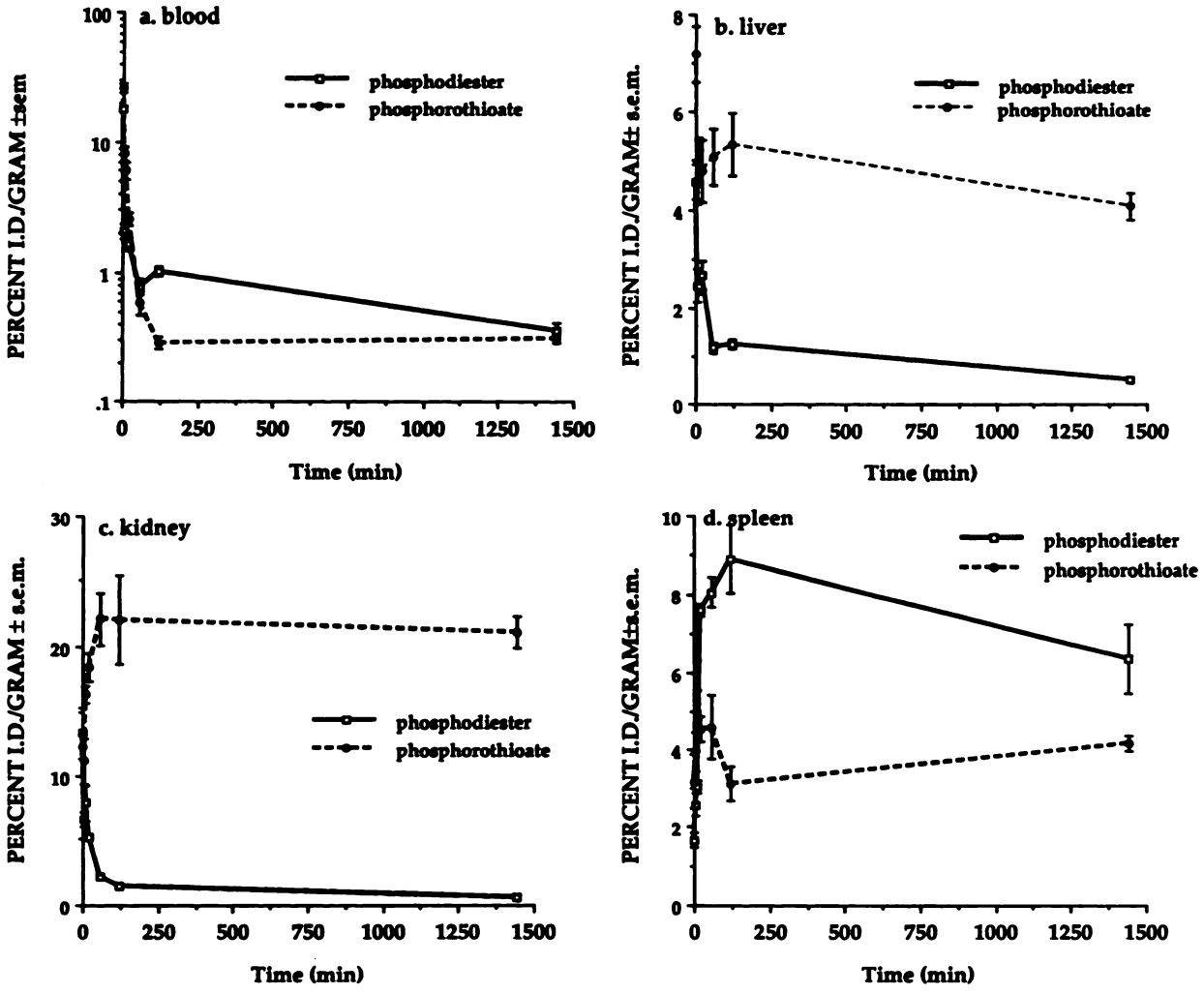

Fig. 5. Biodistribution of 6 mg/kg ^{3}H-phosphodiester and ^{3}H-phosphorothioate oligonucleotides in mice.

and 15.9 hr, respectively. These values are questionable, however, because of the rapid metabolism.

The biodistribution of ^{3}H-phosphodiester oligonucleotide suggests that little of this molecule is taken up by tissues. Inulin is a noncharged polysaccharide about the size of an oligonucleotide (5 kDa) that is freely diffusible in body water and is cleared rapidly by glomerular filtration (16). After intravenous injection, [^{3}H]inulin was rapidly cleared from the blood and the rest of the body, with accretion only by the kidney. Being a freely diffusible compound, inulin had an organ to blood ratio of 1:1 for all organs (except kidney) (Fig. 6) 2 hr after injection. The elevated kidney to blood ratio reflects the mode of elimination of inulin (renal clearance). After the injection of ^{3}H-phosphodiester oligonucleotide, organ to blood ratios for all organs, except spleen, were essentially 1:1, indicating that the oligonucleotide, like inulin, diffused freely without either binding to tissue or accumulating within tissues. Splenic values of approximately 8:1 suggest that this organ may be a site of

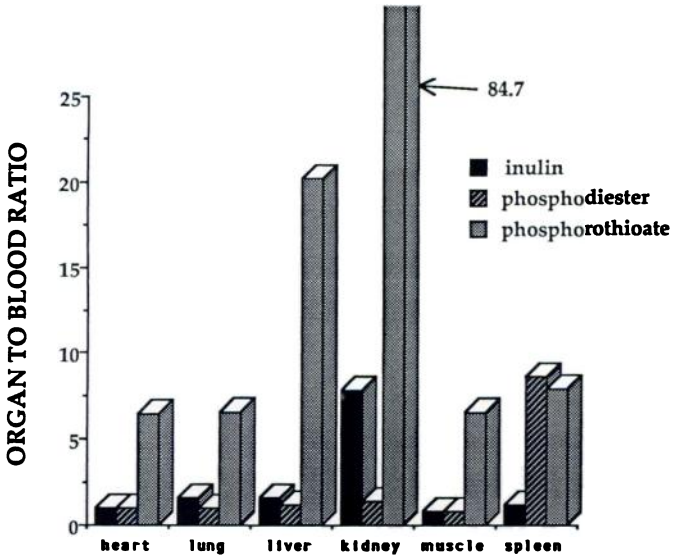

Fig. 6. Organ to blood ratio calculated from the biodistribution data collected 2 hr after injection.

accretion of either the oligonucleotide or, more likely, its metabolites.

Despite the rapid clearance of ^{3}H from the blood after an intravenous injection of phosphorothioate oligonucleotide, high levels were found in the liver, kidney, and most other tissues at all time points studied (Fig. 5; Table 2). The spleen did not appear to accumulate ^{3}H after the injection of ^{3}H-phosphorothioate oligonucleotide (Fig. 5d). The accretion of ^{3}H from the injected phosphorothioate oligonucleotide occurred in all tissues and resulted in the retention of substantial amounts of the ID. Thirty-five percent of the ID (or 70% of the 1-min recovered dose) could be found in the mice 24 hr after injection (Table 2). The organ to blood ratios found after the injection of ^{3}H-phosphorothioate oligonucleotide were elevated in every organ studied, indicating accumulation of either the oligonucleotide or its metabolites. The organ to blood ratio was most striking for liver (23.3:1) and kidney (84:1). Estimates of volume of distribution and blood half-life for total-body elimination of the phosphorothiate oligonucleotide are 218.9 ml and 66.0 hr, respectively.

HPLC analysis of liver, kidney, and spleen. To determine the molecular nature of the tritiated species present, livers, spleens, and kidneys were homogenized in the presence of GIT and extracted with phenol/chloroform. The aqueous material remaining in the aqueous fraction was analyzed by paired-ion chromatography. In tissue samples taken from animals sacrificed 1 min after injection of ^{3}H-phosphodiester oligonucleotide, ^{3}H was found only in peaks that coeluted with either 5-methyl-2′-deoxydeoxyctidine or thymidine (Fig. 7). There was no evidence for the presence of intact oligonucleotide, thus indicating its rapid and complete metabolism.

In contrast to these results were those from the HPLC analysis of kidneys and livers taken from animals injected with ^{3}H-phosphorothioate oligonucleotide (Fig. 8). (Radioactivity in the spleen could not be analyzed due to the very small amount accumulated.) Immediately after injection, ^{3}H could be seen only in the intact oligonucleotide peak. With time this peak broadened and shifted to the left. At no time could peaks that coeluted with either 5-methyl-2′-deoxydeoxyctidine or thymidine be seen, indicating the lack of metabolism to the monomer. Twenty-four hours after injection, approximately 20% of the counts were associated with the full length material, whereas the bulk of the radioactivity was associated with shorter oligonucleotides. These results are consistent with the expected metabolism of an oligonucleotide labeled in the middle, in

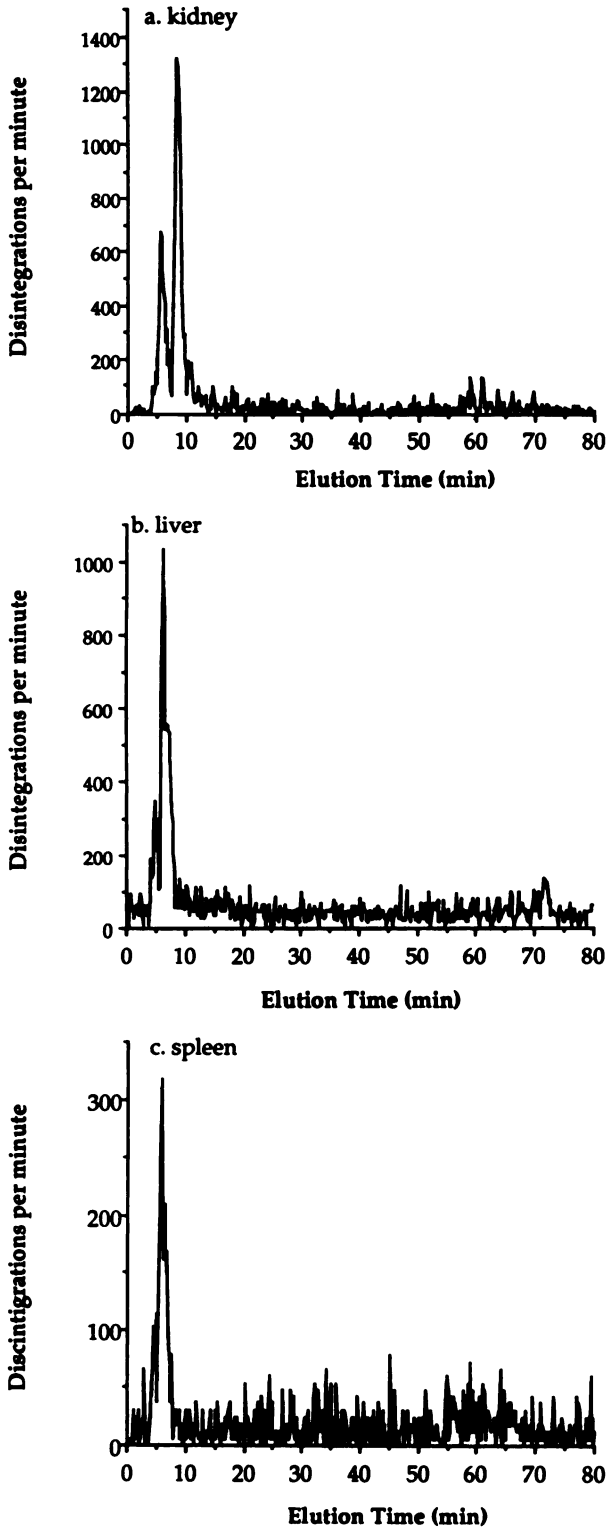

Fig. 7. Paired-ion chromatographic analysis of organ extracts taken from mice injected 1 min previously with ^{3}H-phosphodiester oligonucleotide.

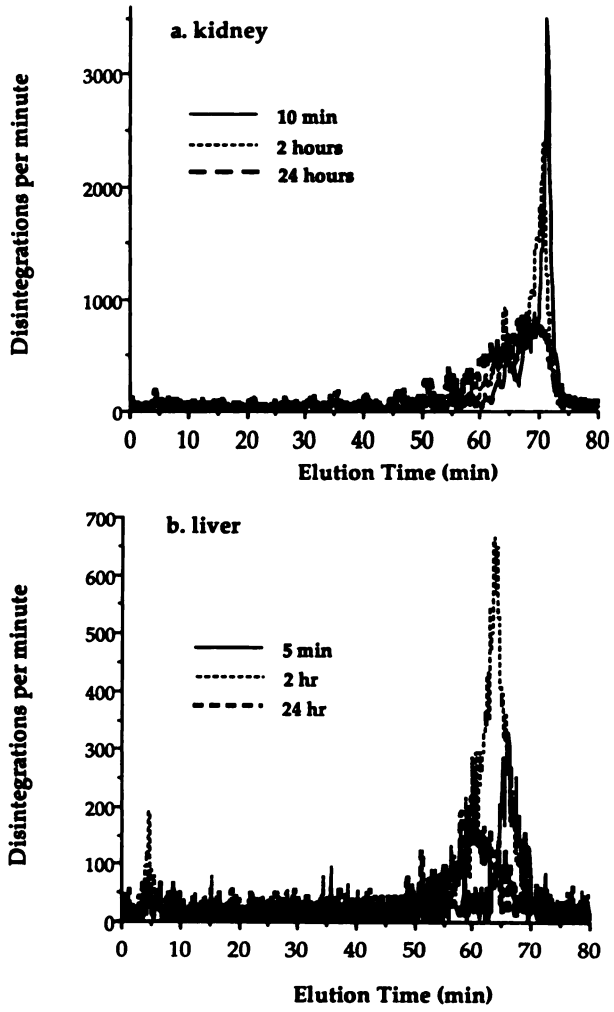

Fig. 8. Paired-ion chromatographic analysis of organ extracts taken from mice injected with ^{3}H-phosphorothioate oligonucleotides.

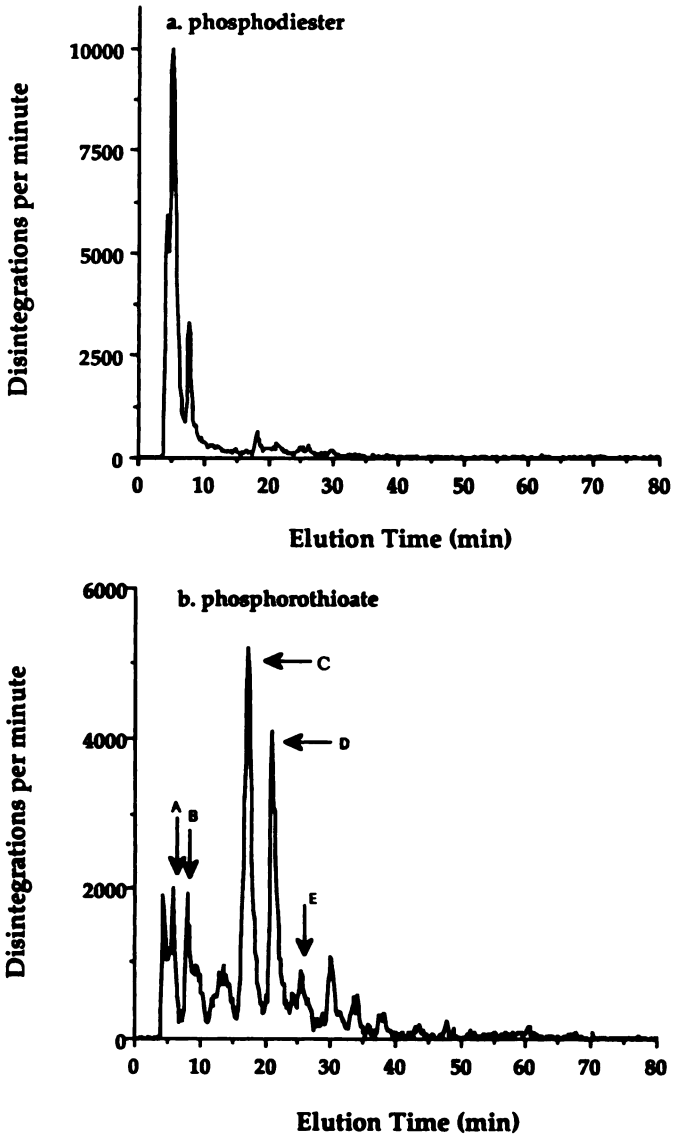

Fig. 9. Paired-ion chromatographic analysis of 24-hr pooled urine collected from animals injected with phosphodiester (a) or phosphorothioate (b) oligonucleotides. *Peaks A* and *B* coelute with 5-methyldeoxycytidine and thymidine, respectively. *Peaks C*, *D*, and *E* have retention time appropriate for nucleosides, mononucleotide phosphorothioates, dinucleotide phosphorothioates, and trinucleotide phosphorothioates, respectively.

which the bases have been slowly removed from the 3′ and/or 5′ ends.

HPLC analysis of urine. HPLC analysis of the urine collected during the 24 hr after intravenous injection of oligonucleotides is shown in Fig. 9. After injection of phosphodiester essentially all of the ^{3}H found in urine eluted with the monomers, indicating complete breakdown of the oligonucleotide (Fig. 9a). This result was not surprising, given the analysis of organ content described above. No metabolites were seen after *in vitro* incubation of oligonucleotide in mouse urine for 24 hr. This indicates that the metabolic products were produced *in vivo* (data not shown) and not during the collection process. The lack of intact oligonucleotide in urine suggests that glomerular filtration did not occur to any significant extent before the oligonucleotide was completely broken down.

A different pattern was seen when the 24-hr urine samples from animals injected with phosphorothioate oligonucleotide were analyzed (Fig. 9b). As with the phosphodiester oligonucleotide, the urine contained 5-methyl-2′-deoxyctidine (Fig. 9b, *peak A*) and thymidine (Fig. 9b, *peak B*) and no full length oligonucleotide. In the case of the phosphorothioate oligonucleotide, however, many other metabolites were also present. Comparison of the metabolites with various nucleotide standards by HPLC suggests that the major urinary metabolites are nucleosides, mononucleotide phosphorothioates, dinucleotide phosphorothioates, and trinucleotide phosphorothioates (Fig. 9b, *peaks C*, *D*, and *E*, respectively).

These results indicated that, despite the substitutions in the backbone, complete breakdown of the oligonucleotide did occur to some extent. Again, the lack of any intact oligonucleotide in the urine suggests that glomerular filtration did not occur, possibly due to binding to serum proteins. Incubation of phosphorothioate oligonucleotide with mouse urine for 24 hr at room temperature did not result in oligonucleotide metabolism (data not shown). Because the radiolabel was placed on a single base within the oligonucleotide, the finding of monomers, dimers, and trimers in the urine is consistent with the slow but eventually complete degradation of the phosphorothioate oligonucleotide.

Serum binding. To assess the role played by binding to

serum albumin, the binding of oligonucleotides to human albumin was determined. For comparison, parallel binding studies were carried out with a drug known to have a high percentage of albumin binding (digitoxin) and one with a known low percentage of albumin binding (digoxin). To conserve unlabeled oligonucleotide, the concentrations of both human serum albumin and drug were reduced by a factor of 10 from those usually used (17). As can be seen in Table 3, the relative binding of digitoxin and digoxin was high and low, respectively, as expected (18). The phosphorothioate oligonucleotide demonstrated a level of binding essentially equal to that of digitoxin, whereas the phosphodiester oligonucleotide showed less specific binding. Similar results have been reported by Ghosh *et al.* (19). The serum albumin binding of the phosphorothioate can partially account for the lack of intact oligonucleotide found in urine.

Kidney autoradiography. Autoradiographs of kidneys taken from mice injected with ^{3}H-oligonucleotides are shown in Fig. 10. Five minutes after injection of phosphodiester, radioactivity could be seen throughout the kidney, with maximal concentrations in the medulla (Fig. 10A). The pattern suggests distribution in the cortical blood pool, accompanied by rapid urinary excretion (medulla). As was seen in the biodistribution studies (Fig. 5c), 2 hr after injection there was a major reduction in the amount of radiolabel left in the kidney. Urinary clearance appears to be complete at this time, as indicated by the lack of radiolabel found in the medulla.

This general blood pool pattern was not detected after injection of ^{3}H-phosphorothioate (Fig. 10B). At times of maximum radiolabel content (2 hr and 24 hr), as determined by the biodistribution studies (Fig. 5c), a discrete binding pattern was seen in the cortex, with complete lack of radioactivity in the medulla. There is little evidence of clearance from the kidney over time, and the lack of medullary activity suggests that renal clearance was markedly less than that seen with the phosphodiester.

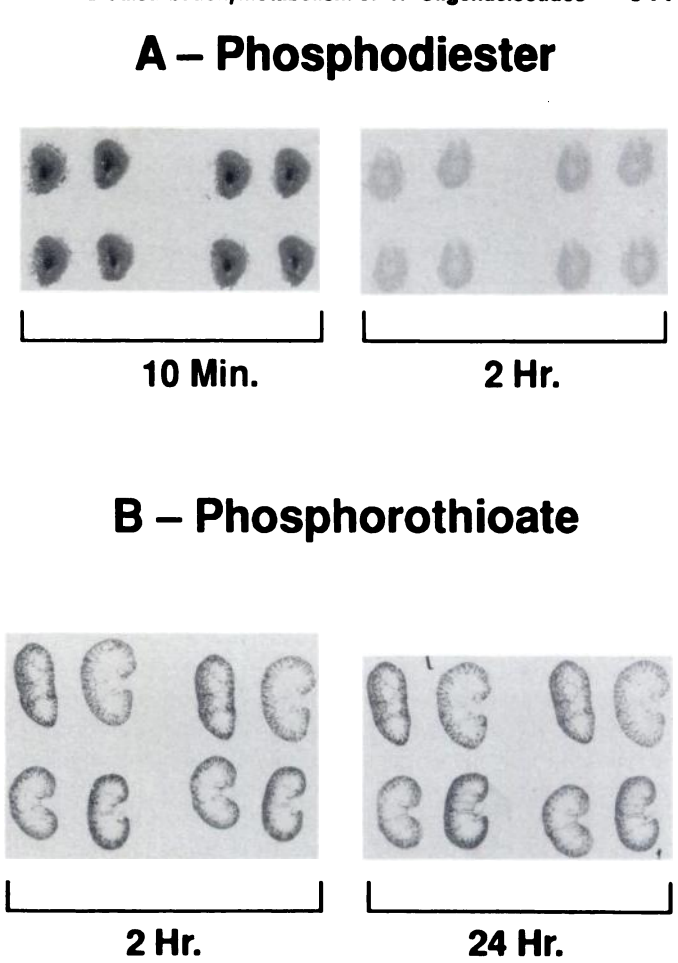

Fig. 10. Autoradiographic analysis of mouse kidneys after injection of 6 mg/kg ^{3}H-phosphodiester oligonucleotide (A) or phosphorothioate oligonucleotide (B) for the times indicated.

Discussion

The potential specificity and degree of informational content are the major attractions of oligonucleotide-based antisense drugs (1–3). For these entities to become efficacious drugs, first they must be stable to degrading enzymes present in the blood, in the interstitium, and within cells. Second, they must accumulate in sufficient quantity within their target organs and cells to elicit a pharmacological response. Of the available modified oligonucleotides, only a few have been studied *in vivo* (6–10). The use of different lengths, sequences, labeling methodologies, and species, as well as the lack of comparisons with unmodified oligonucleotides, has made it difficult to draw any conclusions as to their relative merit for drug development. This study presents a comparison of the pharmacokinetics and metabolism of two oligonucleotides of the same length and sequence, radiolabeled at the same base and differing only in their backbone modification.

TABLE 3
Binding to human serum albumin

Drug (10^{-6} M)	Specific binding
	%
Digitoxin	56
Digoxin	4
Phosphodiester oligonucleotide	30
Phosphorothioate oligonucleotide	65

Use of radiolabeled oligonucleotide allowed the rapid analysis of organ content and secondary analysis of radiolabeled metabolites. Previous studies that used ^{32}P-end-labeled oligonucleotides were affected by the rapid release of label by exonucleases and phosphatases, resulting in conflicting and potentially misleading results (6–8). In addition, the existing methods for tagging oligonucleotides with radionuclides suffer other disadvantages, e.g., short half-life of the radioisotope, the presence of label at multiple sites, the danger of high energy β-emissions, and a requirement for waiting 10 half-lives before disposing of waste. A method for tagging oligonucleotides with tritium at a single internal cytosine was developed that eliminates or minimizes these problems. In this method, *Hha*I methylase is used to deliver a tritiated methyl group from [^{3}H]SAM specifically to the first cytosine in the recognition site of this methylase, GCGC. Oligodeoxyribonucleotides labeled by this method have the following advantageous properties for biodistribution studies. (a) They are stable for several months at −20°. (b) Analysis of metabolites is simplified because only a single site in the oligonucleotide is labeled. (c) Use of tritium as the radioactive label is safer and simplifies waste disposal. (d) Phosphodiesters and phosphorothioates can be labeled by the same strategy. (This strategy is also compatible with end-modifications intended to block degradation or stimulate uptake.) (e) Most

importantly, metabolic processes do not release the radioactive label from the oligonucleotide until it has been extensively degraded and is unlikely to be capable of exerting an antisense effect. Although there are several potential drawbacks to the method, they did not prove to be significant limitations. First, the oligonucleotide was required to contain a GCGC site for recognition by *Hha*I methylase. Because the purpose of this study was to examine the pharmacokinetics of oligonucleotides in general (and not a specific antisense oligonucleotide), this requirement was not considered a limitation. For future needs, use of other nucleic acid methylases could permit tagging at other sites. Second, oligonucleotides are conventionally analyzed by gel electrophoresis, a separation medium that makes detection of low energy emission from tritium difficult to detect. (After gel electrophoresis, about 100,000 dpm of tritiated oligonucleotide could be detected by fluorography.) This problem was negated by the development of a paired-ion HPLC system, which can substitute for gel electrophoresis.

To complement the biodistribution studies, which follow only the radiolabel, tissue samples were extracted and analyzed by paired-ion HPLC to determine the molecular nature of the radiolabel. Fortunately, ion-paired HPLC proved capable of providing separations similar to those normally obtained from gel electrophoresis. These techniques were able to resolve unmetabolized oligonucleotides from their shortened metabolic products. Resolution varied somewhat with the sample being analyzed. Under optimal conditions the 20-nucleotide phosphodiester and a 19-nucleotide degradation product eluted with nearly base-line separation. When smaller samples were analyzed, separation could diminish to the point where the 19-nucleotide degradation product was detected only by an increased width of the 20-nucleotide oligonucleotide peak. The phosphorothioate oligonucleotide gave a slightly broader peak than did the phosphodiester oligonucleotide. Because both 19-nucleotide and 20-nucleotide oligonucleotides should be capable of exerting similar antisense effects, this degree of resolution was judged adequate. The rapid degradation (probably by exonucleases) of the phosphodiester oligonucleotide and the slower metabolism of the phosphorothioate oligonucleotide were easily monitored.

One unique feature of these studies was the inclusion of [^{3}H] inulin as a control "drug" of similar molecular weight that is not taken up or bound by tissues and the use of the percentage of ID/organ values to calculate the percentage of ID recovered. Inulin is an uncharged polysaccharide of approximately 5 kDa, whose biodistribution and pharmacokinetics are well known. As expected, it was rapidly cleared from the blood and the rest of the body, with accretion only in the kidney. A second unique feature was calculation of the total recovered dose of radiolabel, thus allowing for a greater understanding of the fraction of the oligonucleotide dose remaining in the mice during the study.

The results indicated that the phosphodiester oligonucleotide was cleared rapidly from the blood, in a manner similar to that of inulin. The organ to blood ratio values of 1:1 indicated that both molecules diffused rapidly into all organs. Using paired-ion HPLC, very little intact oligonucleotide was found in any organ 1 min after injection into the mice, indicating that the oligonucleotide was rapidly metabolized to its mononucleotides. (Elapsed time from injection to removal and freezing of the organ was approximately 5 min.) Degradation *in vivo* was considerably faster than that seen in mouse serum. Autoradiography of the kidney indicated distribution in the blood pool, rapid accumulation in urine, and rapid clearance from the organ. The speed and extent of the splenic uptake of radiolabel from the phosphodiester oligonucleotide were similar to those of uptake of thymidine and 5-methyl-2′-deoxycytidine, suggesting that splenic uptake from the oligonucleotides was most likely due to the ability of the spleen to scavenge the radiolabeled metabolites. Radiolabel cleared the body, leaving only a small percentage of the dose in the animal after 24 hr. HPLC studies indicated that few, if any, of the counts present in the mouse at any time point were attached to oligonucleotides capable of exerting an antisense effect. As expected from these findings, essentially all of the radiolabel found in the urine was in the form of small metabolites.

The data generated using the phosphorothioate oligonucleotide contrast sharply with those for the phosphodiester. Despite the higher degree of serum albumin binding, the phosphorothioate oligonucleotide clearance rate from the blood was slightly more rapid than that of the phosphodiester. All organs, except blood, showed a rapid and prolonged accretion of the radiolabel. Organ to blood ratios were at least 5:1 in the tissues examined, indicating that the radiolabel was accumulated by various organs. Absolute organ uptakes were elevated for the duration of the experiment. (The mechanisms underlying this rapid accretion of a highly protein-bound compound are now under study.) The greatest uptake was by the kidney and liver. Two hours after injection, the radiolabel in these organs was mostly associated with the intact oligonucleotide. By 24 hr after injection, the fraction of the intact oligonucleotide was markedly reduced (with a concomitant increase in shorter oligonucleotides). The size distribution of these shorter oligonucleotides is consistent with metabolism predominantly by exonucleases. Metabolism appeared to occur at a faster rate in the liver than in the kidney. Autoradiographs of kidney slices suggested that binding within the kidney was to discrete anatomical moieties within the kidney cortex, perhaps those associated with the glomerulus and the loop of Henle, rather then to the collecting ducts. Mononucleotides and very short oligonucleotides were never detected in any organ, but they were present in urine. Because the radiolabel was located in the middle of the molecule, these small labeled degradation products would not be observed until the oligonucleotide was almost completely degraded. These small metabolites were probably removed so rapidly from the organs that no accumulation was seen. The slow *in vivo* metabolism reflected the slow degradation seen in mouse serum. This evidence for very slow metabolism plus slow clearance from the mouse suggests that large portions of the dose of phosphorothioate oligonucleotide remain intact throughout the animal for an extended time period.

de Smidt *et al.* (6) have reported on the plasma half-life and organ distribution, after intravenous injection into rats, of a 16-nucleotide phosphodiester oligonucleotide terminally labeled with ^{32}P. Information on this oligonucleotide was limited because this was the control moiety for the study of an oligonucleotide-cholesterol conjugate. Those authors reported rapid plasma clearance (calculated to be very similar to what we saw in the mouse), with radioactivity being rapidly processed by the kidney to the urine.

Three published reports deal with the biodistribution and fate of phosphorothioate oligonucleotides (7, 8, 10). Agrawal *et al.* (10) injected into mice, intravenously, a 20-nucleotide oli-

gonucleotide uniformly labeled in the backbone with ^{35}S. Those studies lacked a comparison with the unmodified oligonucleotide and relied on data from a single mouse at each time point. Their results were very similar to those reported here. ^{35}S rapidly cleared from the blood and accumulated in most organs, with the greatest accumulation being found in the kidney. Breakdown was slow in plasma but more rapid in liver and kidney. Only 10–15% degradation was seen in most organs. Thirty percent of the ID was excreted via the kidney in 24 hr and degradation was attributed to exonuclease activity. The data in this report and that of Agrawal *et al.* (10) conflict with data reported by both Goodarzi *et al.* (7) and Inagaki *et al* (8). Using either 15- or 20-mers terminally labeled with ^{32}P, injected intravenously into mice, they reported that the largest organ accumulation was in the liver and that rapid degradation (50% reduction in length in 30 min) occurred. Because their data were reported only as cpm, further comparisons were not possible. Some of the differences could be explained by the rapid removal of the terminal label, rather then extensive breakdown of the oligonucleotide.

The studies presented here are the first *in vivo* direct comparison of a phosphodiester oligonucleotide with a phosphorothioate oligonucleotide, of the same length, sequence, and labeling technique. Use of [^{3}H]inulin and paired-ion HPLC analysis allows for a more complete evaluation of the therapeutic potential of each backbone modification. The data generated suggest that the phosphodiester oligonucleotide would have limited therapeutic utility due to its low organ accretion and rapid metabolism. In contrast, the stability and organ distribution of the phosphorothioate oligonucleotide imply that oligonucleotides bearing this modification may have therapeutic potential.

Acknowledgments

The authors wish to thank Dr. William Herblin and Shelia Diamond for producing and analyzing the autoradiographs and Ashley Beasley for her help with the organ extractions and paired-ion chromatographic analysis.

References

1. Uhlmann, E., and A. Peyman. Antisense oligonucleotides: a new therapeutic principle. *Chem. Rev.* **90**:543–583 (1990).
2. Cohen, J. S. Oligonucleotides as therapeutic agents. *Pharmacol. Ther.* **52**:211–225 (1992).
3. Cohen, J. S. Phosphorothioate oligonucleotides, in *Antisense Reseach and Applications* (S. T. Crooke and B. Lebleu, eds.). CRC Press, Boca Raton, FL, 205–221 (1993).
4. Stein, C. A. Antisense oligodeoxynucleotides: promises and pitfalls. *Leukemia* **6**:967–974 (1992).
5. Stein, C., and J. Cohen. Phosphorothioate oligodeoxynucleotide analogs, in *Oliogodeoxyncleotides: Antisense Inhibitors of Gene Expression* (J. Cohen, ed.). Macmillan, London, 97–117 (1989).
6. de Smidt, P. C., T. L. Doan, S. de Falco, and T. J. C. van Berkel. Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acids Res.* **19**:4695–4700 (1991).
7. Goodarzi, G., M. Watabe, and K. Watabe. Organ distribution and stability of phosphorothiated oligodeoxyribonucleotides in mice. *Biopharm. Drug Dispos.* **13**:221–227 (1992).
8. Inagaki, M., K. Togawa, B. I. Carr, K. Ghosh, and J. S. Cohen. Antisense oligonucleotides: inhibition of liver cell proliferation and *in vivo* disposition. *Transplant. Proc.* **24**:2971–2972 (1992).
9. Chem, T.-L., P. S. Miller, P. O. Ts'o, and O. M. Colvin. Disposition and metabolism of oligodeoxynucleoside methylphosphonate following a single iv injection in mice. *Drug Metab. Dispos.* **18**:815–818 (1990).
10. Agrawal, S., J. Temsamani, and J. Y. Tang. Pharmacokinetics, biodistribution and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA* **88**:7595–7599 (1991).
11. Boutorine, A. S., T. L. Doan, J. P. Battionii, D. Mansuy, D. Dupre, and C. Helene. Rapid routes of synthesis of chemically reactive and highly radioactive labeled *alpha*- and *beta*-oligonucleotide derivatives for *in vivo* studies. *Bioconjugate Chem.* **1**:350–356 (1990).
12. Whitesell, L., D. Geselowitz, C. Chavany, B. Fahmy, S. Walbridge, J. R. Alger, and L. M. Neckers. Stability, clearance, and disposition of intraventricularly administered oligonucleotides: implication for therapeutic application within the central nervous system. *Proc. Natl. Acad. Sci. USA* **90**:4665–4669 (1993).
13. Zendegui, J. G., K. M. Vasquez, J. H. Tinsley, D. J. Kessler, and M. E. Hogan. *In vivo* stability and kinetics of absorption and disposition of 3′-phosphopropyl amine oligonucleotides. *Nucleic Acids Res.* **20**:307–314 (1992).
14. Hobbs, F. W., and J. A. Yarem. Denaturing HPLC purification of tritylated oligonucleotides using tetraethylammonium hydroxide. *BioTechniques* **14**:584 (1993).
15. Fasman, G. D., ed. *Handbook of Biochemistry and Molecular Biology*, Vol. 1. CRC Press, Cleveland, OH, 589 (1976).
16. Bourne, G. R., and H. E. Barber. The pharmacokinetics of inulin and urea: a comparison of the dose eliminated from a compartmental model and that eliminated in urine. *J. Pharm. Pharmacol.* **24**:532–537 (1971).
17. Kurz, H., H. Trunk, and B. Weitz. Evaulation of methods to determine protein binding of drugs: equilibrium dialysis, ultrafiltration, ultracentrifugation, and gel filtration. *Arzneim. Forsch.* **27**:1373–1390 (1977).
18. Hoffmann, B. F., and J. Bigger, Jr. Digitalis and allied cardiac glycosides, in *The Phramacological Basis of Therapeutics* (A. G. Gilman, T. W. Rall, A. S. Nie, and P. Taylor, eds.), Ed. 8. Pergamon Press, New York, 814–839 (1990).
19. Ghosh, M. K., K. Ghosh, and J. S. Cohen. Phosphorothioate-phosphodiester oligonucleotide co-polymers: assessment for antisense applications. *Anti-Cancer Drug Design* **8**:15–32 (1993).

Send reprint requests to: Howard Sands, Lexin Pharmaceutical Co., 111 Rock Road, Horsham, PA 19044.