0026-895X/95/030636-10$3.00/0

MOLECULAR PHARMACOLOGY, 47:636–646, 1995.

Biodistribution and Metabolism of Internally ^{3}H-Labeled Oligonucleotides. II. 3′,5′-Blocked Oligonucleotides

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Received August 29, 1994; Accepted December 9, 1994

SUMMARY

The pharmacokinetics and metabolism of four radiolabeled phosphodiester oligonucleotides with 3′- and 5′-blocked ends were studied in mice and compared with previously studied, unblocked, all-phosphodiester and all-phosphorothioate oligonucleotides. The radiolabel was a tritiated methyl group enzymatically attached at an internal cytidine. The ends of the blocked phosphodiester oligonucleotides were protected by cyclization or by incorporation of either phosphorothioate or methylphosphonate linkages. Although these modifications protected the blocked oligonucleotides from degradation by exonucleases present in mouse serum, degradation initiated by endonucleases was 50% complete in 0.5–5 hr. After intravenous injection, the blocked oligonucleotides were much less stable than the all-phosphorothioate oligonucleotide and only marginally more stable than the previously studied, unblocked phosphodiester oligonucleotide. Even a "chimeric" blocked oligonucleotide with 16 phosphorothioate linkages and eight contiguous phosphodiester linkages was rapidly degraded. Despite the favorable serum binding, tissue accumulation, and stability observed with phosphorothioate oligonucleotides, these properties did not provide the chimeric oligonucleotide access to a compartment where its phosphodiester linkages were stable. In other respects, the blocked and chimeric phosphodiester oligonucleotides also resembled the unblocked phosphodiester oligonucleotide; radiolabel was cleared rapidly from the blood, there was little evidence of tissue accumulation, high performance liquid chromatographic analysis of tissue extracts showed extremely rapid degradation to mononucleotides, and only mononucleotide metabolites were present in urine. In summary, blocked phosphodiester oligonucleotides are rapidly attacked by endonucleases present in mice. Unless this problem is less serious in primates, such blocked oligonucleotides will be relatively unattractive candidates for drug development.

Antisense oligonucleotides designed to block synthesis of specific proteins associated with disease states have been hailed as the next generation of drugs (1–3). A major limitation of these compounds is the susceptibility of their phosphodiester linkages to degradation by exo- and endonucleases. This problem can be reduced or eliminated by replacing phosphodiester linkages with PS (4) or MP linkages (5). Unfortunately, extensive use of these modified linkages reduces (a) the affinity of an oligonucleotide for an mRNA target (6) and (b) its ability to permanently inactivate a specific mRNA by mediating mRNA cleavage by RNase H (7). Séveral reports demonstrate that 3′-exonucleases are the major enzymes responsible for oligonucleotide degradation in serum and in media used for cell culture experiments (8–10). Various strategies for blocking the 3′-end or 3′- and 5′-ends of phosphodiester oligonucleotides have been reported to significantly enhance oligonucleotide stability in serum or antisense activity in cell culture experiments (11–13). Endonucleases, however, have been implicated in the degradation of blocked oligonucleotides inside cells (14–16). The efficacy of these blocking strategies and the relative importance of degradation by exo- and endonucleases have not been conclusively established in animal models. To our knowledge, the pharmacokinetics and metabolism of only one blocked oligonucleotide, a phosphodiester with a 3′-phosphopropyl amine, have been studied in animals (17). In that study, Zendegui *et al.* (17) concluded that 3′-blocked oligonucleotides are relatively stable *in vivo* and exhibit biodistribution appropriate for use as therapeutic agents.

Herein, the pharmacokinetics and metabolism of four blocked phosphodiester oligonucleotides were studied in mice by methods used previously to study homogeneous phosphodiester and PS oligonucleotides (18). The ends of these oligonucleotides were protected from degradation by (a) PS linkages ("PS-blocked" and "chimeric" oligonucleotides), (b) MP linkages ("MP-blocked" oligonucleotide), or (c) cyclization (Fig. 1). A cytidine in the central phosphodiester region was

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ABBREVIATIONS: PS, phosphorothioate; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonate; MP, methylphosphonate.

A. Unmodified phosphodiester oligonucleotide (20 bases):

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5'- TpCpApTpGpCpTpCpApTpGpCpGpCpTpCpApTpGpC -3'
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Phosphorothioate oligonucleotide (20 bases):

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5'- TsCsAsTsGsCsTsCsAsTsGsCsGsCsTsCsAsTsGsC -3'
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"PS-blocked" phosphodiester oligonucleotide (20 bases):

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5'- TsCsApTpGpCpTpCpApTpGpCpGpCpTpCpApTsGsC -3'
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"MP-blocked" phosphodiester oligonucleotide: (20 bases):

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5'- TmCmApTpGpCpTpCpApTpGpCpGpCpTpCpAmTmGmC -3'
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"Chimeric" oligonucleotide (25-bases):

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5'- TsCsApTpGpCpGpCpTpCpAsTsCsAsTsGsGsCsCsTsCsAsTsGsC -3'
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Circular phosphodiester oligonucleotide (34 bases):

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5'-    ApCpTpTpTpCpTpTpCpTpTpCpTpTpGpC    -3'
     p                                 p
   C                                     G
     p                                 p
3'-    ApCpTpTpTpCpTpTpCpTpTpCpTpTpTpC    -5'
```

Fig. 1. A, Oligonucleotides studied in this and previous work (18). *p*, phosphodiester linkage; *s*, PS linkage; *m*, MP linkage. *Underlined cytidines* in the *Hha*I methylation sites (GCGC) were tagged at the 5-position with a tritiated methyl group. B, Alignments of the PS (*first, from top*), chimeric (*second*), and PS-blocked phosphodiester oligonucleotides (*third* and *fourth*), showing how the chimeric oligonucleotide was designed. A radiolabeled nine-nucleotide phosphodiester segment (in *bold*) from the PS-blocked phosphodiester oligonucleotide was inserted into the PS oligonucleotide. Except for switching two bases to inactivate a second *Hha*I methylation site (in *italics*), the 14-nucleotide PS 3′-region is identical in both the chimeric and PS oligonucleotides. Five bases at the 5′-end and two bases at the 3′-end of the blocked and chimeric oligonucleotides are also identical.

B. Alignment of phosphorothioate (top), chimeric (second), and PS-blocked oligonucleotides (bottom two).

```
          TsCsAsT.........sGsCsTsCsAsTsGsCsGsCsTsCsAsTsGsC
          TsCsApTpGpCpGpCpTpCpAsTsCsAsTsGsGsCsCsTsCsAsTsGsC
TsCsApTpGpCpTpCpApTpGpCpGpCpTpCpApT.................sGsC
          TsCsApTpGpCpTpCp.........ApTpGpCpGpCpTpCpApTsGsC
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enzymatically tagged with a [^{3}H]methyl group. In contrast to the conclusions reached by Zendegui *et al.* (17), our results suggest that end protection strategies failed to adequately improve *in vivo* stability of phosphodiester oligonucleotides and did not afford attractive drug candidates.

Materials and Methods

Many of the methods used have been described in detail in a previous publication (18) and are summarized below. Preparation of blocked oligonucleotides is described in detail.

Preparation of Oligonucleotides

Synthesis. All oligonucleotides were 2′-deoxyribonucleotides and were synthesized, purified, converted to sodium salts, and quantitated as described previously (18). MP linkages were prepared using methyl phosphonamidites (JBL Scientific, San Luis Obispo, CA), following the manufacturer's recommendations. The oligonucleotide with MP linkages was cleaved and deprotected using the two-step procedure (ammonia followed by ethylenediamine) of Hogrefe *et al.* (19). A 3′-phosphorylated linear phosphodiester oligonucleotide, 5′-TTCTTTCACACTTTCTTCTTCTTGCGCTTTCTTCp-3′, was synthesized using Phosphate-ON phosphoramidite (Glen Research, Sterling, VA) and was cyclized by the method of Shabarova and co-workers (20, 21). This oligonucleotide (50 nmol) was annealed over 1.5 hr to a template phosphodiester oligonucleotide complementary to both ends, 5′-AAAGAAGAAGAA-3′, in 900 μl of solution containing 0.28 M MES, pH 7.0, and 22 mM $MgCl_2$, and was chemically ligated at 0° by addition of 100 μl of 10 M cyanogen bromide in acetonitrile. After 10 min, the oligonucleotide mixture was isolated by ethanol precipitation. Approximately 70–80% of the linear precursor was cyclized under these conditions. The circular product (which migrates about 10% more slowly than its linear precursor) obtained from 60 identical reactions was separated from other oligonucleotides by preparative polyacrylamide gel electrophoresis and was isolated by dialysis. The circular nature of the product was unambiguously proven by (a) sequencing across the ligation site and (b) digestion with *Hha*I endonuclease to a linear 34-base oligonucleotide (rather than 25- and 9-base fragments). Additional preparative information and complete characterization of this oligonucleotide will be described elsewhere.[2]

^{3}H-Labeling. The PS-blocked, MP-blocked, and chimeric oligonucleotides were tritiated as described previously (18). Briefly, the oligonucleotide (27.5 nmol), a complementary 10-mer with a methylated *Hha*I site (27.5 nmol), *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 mCi = 13.6 nmol), and *Hha*I methylase (2500 units) were incubated at 37° overnight in a total of 275 μl of buffer. After thermal denaturation of the enzyme, the tritiated oligonucleotide was purified by gel filtration, preparative polyacrylamide gel electrophoresis, and Sep-Pak chromatography. Approximately 50% of the starting oligonucleotide was recovered, with a specific activity of 10–15 μCi/nmol. The circular oligonucleotide was tritiated as described above, except that the complementary strand was a 16-mer (5′-AAGAAAGcGCAAGAAG-3′; c denotes 2′-deoxy-5-methylcytidine). None of the end modifications interfered significantly with enzymatic methylation.

Biodistribution

Radiolabeled oligonucleotides (6 mg/kg, containing approximately 5 μCi of ^{3}H) were injected via the tail vein into male CF1 mice weighing approximately 28 g. Animals were sacrificed by CO_2 asphyxiation at the indicated times. Tissue collection and urine collec-

[2] S. P. Ho and Y. Bao, manuscript in preparation.

tion followed procedures reported previously (18). Control experiments established that oligonucleotides were stable in urine and frozen tissue. For the determination of whole-body clearance, the percentage of injected dose recovered was calculated using the assumptions and quench corrections reported previously. One minute after injection (i.e., before significant clearance could occur), 37–71% of the initial dose was detected in the tissues collected for analysis, which totaled about 65% of the mouse by weight. Whole-body clearance (presumably by urine or feces) was assessed by the decrease from these 1-min values.

Tissue Extraction and Paired-Ion HPLC Analysis

Upon removal, the major metabolic organs (kidney, liver, and spleen) were frozen on dry ice and kept at −20°. Tissue samples were sonicated in lysis buffer containing guanidine isothiocyanate, and the resulting suspension was extracted with phenol/chloroform and centrifuged. Preliminary experiments indicated that >70% of the radioactivity was recovered from a variety of tissue samples, including those with intact or completely degraded oligonucleotide. The paired-ion HPLC analysis separated oligonucleotides and metabolites mostly on the basis of charge, usually with at least partial resolution of oligonucleotides differing by a single charge (18). Although retention times gradually decreased with column use, full length oligonucleotides typically were retained for 60–80 min, whereas mononucleotides were retained for <15 min. The mononucleotide peaks have been shown to be the expected mononucleotide 5-methylcytidine (~5 min) and its deamination product thymidine (~9 min). The HPLC traces in Figs. 2a, 3a, and 4a were corrected for slight decreases in retention time before being superimposed.

Serum Stability Studies

Tritiated oligonucleotides (2 nmol, containing 1 μCi of ^{3}H label) were added to 2 ml of 100% mouse serum (Sigma Chemical Co., St. Louis, MO) and incubated at 37°. (This serum had not been heated to inactivate nucleases). At the indicated times, one tenth of the incubation solution was removed and added to 500 μl of guanidine isothiocyanate. Extraction of the ^{3}H-labeled oligonucleotides and metabolites was carried out according to the procedure reported previously (18).

Results

PS-Blocked Phosphodiester Oligonucleotide

Biodistribution. The results from the biodistribution study with this oligonucleotide are shown in Table 1. Rapid clearance from the blood and most other organs occurred during the 24 hr of the study. Kidney levels were initially high, but the radiolabel was cleared by 24 hr. The only organ that showed sustained accretion of radiolabel was the spleen. Splenic uptake peaked around 2 hr after injection and remained elevated for the next 22 hr. Total radiolabel from the PS-blocked phosphodiester oligonucleotide was cleared rapidly from the mice, leaving only 12% of the injected dose at 24 hr. The biodistribution pattern seen with this PS-blocked phosphodiester oligonucleotide was very similar to that previously seen with an unmodified phosphodiester oligonucleotide with the same sequence but without blocking PS linkages (18).

Serum stability. Oligonucleotides of reduced size and some mononucleotides could be found 2 hr after incubation of the oligonucleotide in normal (i.e., not diluted or heat-inactivated) mouse serum at 37° (Fig. 2a). After 24 hr only the monomer metabolites 5-methyl-2′-deoxycytidine and thymidine were found. Examination of the full time course of degradation (data not shown) suggested that the half-life of intact oligonucleotide was roughly 1 hr.

Tissue metabolism. Paired-ion HPLC analyses of nucleic acids isolated from kidney, liver, and spleen are shown in Fig. 2, b-d. Extremely rapid metabolism of the PS-blocked phosphodiester oligonucleotide was indicated by the fact that only the monomer metabolites could be found in these organs taken from mice sacrificed 1 min after oligonucleotide injection.

Urine analysis. As can be seen in Fig. 2e, urine collected during the first 24 hr after injection of the PS-blocked phosphodiester oligonucleotide contained only monomer metabolites.

MP-Blocked Phosphodiester Oligonucleotide

Biodistribution. Results of the biodistribution experiment with the MP-blocked phosphodiester oligonucleotide are shown in Table 2. Uptake of the MP-blocked phosphodiester oligonucleotide appeared to be greater than that of the PS-blocked phosphodiester oligonucleotide, but the clearance patterns of the two oligonucleotides appeared to be identical. Initial kidney values were high, but the radiolabel was cleared rapidly. As with the PS-blocked

TABLE 1

Biodistribution of the tritiated PS-blocked phosphodiester oligonucleotide

Values are mean ± standard error (n = 5).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
	% of injected dose/g						
Blood	11.32 ± 1.84	2.76 ± 0.21	2.43 ± 0.15	1.65 ± 0.07	1.35 ± 0.10	1.25 ± 0.07	0.92 ± 0.11
Heart	4.18 ± 0.59	2.31 ± 0.18	2.15 ± 0.22	1.69 ± 0.12	1.33 ± 0.14	1.16 ± 0.08	0.74 ± 0.08
Lung	4.95 ± 0.71	2.21 ± 0.18	1.95 ± 0.14	1.47 ± 0.08	1.30 ± 0.14	1.21 ± 0.16	0.88 ± 0.06
Liver	3.31 ± 0.36	2.38 ± 0.18	2.44 ± 0.18	1.98 ± 0.12	1.73 ± 0.19	1.24 ± 0.08	0.76 ± 0.10
Kidney	13.14 ± 1.91	9.43 ± 0.52	6.12 ± 0.23	5.07 ± 0.24	2.60 ± 0.26	1.42 ± 0.07	0.82 ± 0.08
Muscle	0.81 ± 0.10	1.58 ± 0.13	1.75 ± 0.12	1.38 ± 0.10	0.97 ± 0.07	0.93 ± 0.07	0.54 ± 0.05
Skin	1.51 ± 0.24	2.15 ± 0.18	2.08 ± 0.11	1.54 ± 0.10	1.44 ± 0.13	1.81 ± 0.44	0.95 ± 0.10
Spleen	1.56 ± 0.29	2.52 ± 0.30	3.25 ± 0.15	4.41 ± 0.19	9.00 ± 1.40	13.64 ± 3.80	9.73 ± 1.52
				Recovery[a]			
				% of injected dose			
	37.18 ± 4.33	36.41 ± 2.20	37.28 ± 1.25	28.20 ± 1.42	21.68 ± 0.83	23.71 ± 1.55	11.73 ± 0.56

[a] Calculated from percentage of injected dose/organ values.

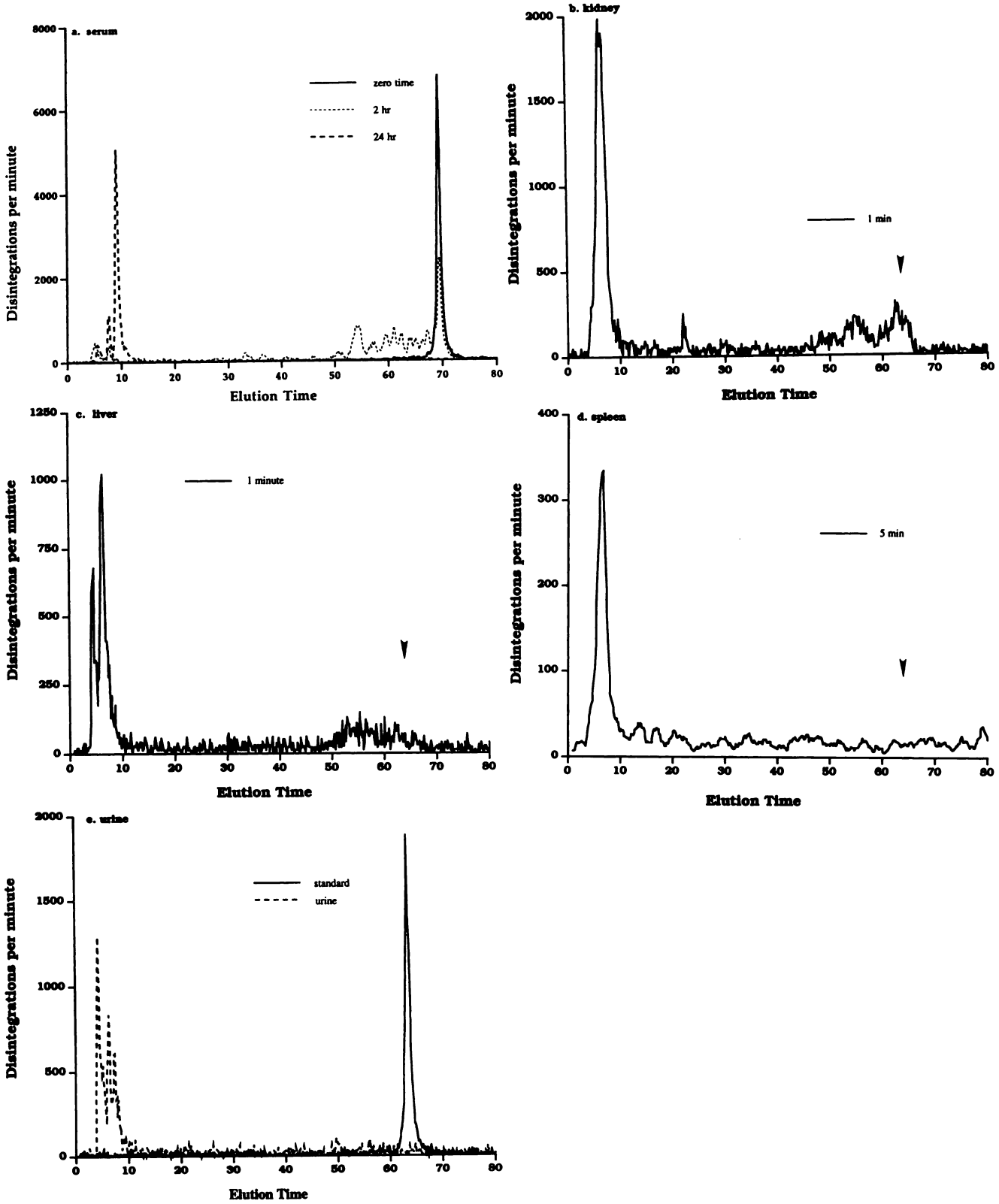

Fig. 2. Paired-ion HPLC of labeled nucleic acids present after addition or injection of the PS-blocked phosphodiester oligonucleotide. a, Serum (*in vitro*); b, kidney; c, liver; d, spleen; e, urine. *Arrowheads in b-d*, retention time of intact oligonucleotide.

phosphodiester oligonucleotide, splenic accretion of radiolabel increased with time for the first 2 hr and remained relatively constant thereafter. The radiolabeled oligonucleotide was cleared rapidly from all other organs. The percentage of the injected dose remaining in the mice was slightly greater after the administration of the MP-blocked phosphodiester oligonucleotide than the PS-blocked phosphodiester oligonucleotide.

TABLE 2
Biodistribution of the tritiated MP-blocked phosphodiester oligonucleotide
Values are mean ± standard error (n = 5).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
				% of injected dose/g			
Blood	14.19 ± 2.44	2.25 ± 0.09	2.04 ± 0.30	2.82 ± 0.36	1.61 ± 0.15	1.72 ± 0.36	1.37 ± 0.08
Heart	6.53 ± 0.51	2.10 ± 0.12	1.83 ± 0.26	2.79 ± 0.33	1.35 ± 0.08	1.66 ± 0.31	1.31 ± 0.07
Lung	7.34 ± 0.99	1.83 ± 0.10	1.76 ± 0.24	2.53 ± 0.31	1.31 ± 0.07	1.59 ± 0.28	1.31 ± 0.10
Liver	5.52 ± 0.29	2.37 ± 0.24	2.67 ± 0.34	4.14 ± 0.48	1.86 ± 0.08	1.89 ± 0.34	1.24 ± 0.06
Kidney	23.52 ± 2.88	7.39 ± 0.48	6.10 ± 0.83	7.93 ± 1.04	3.15 ± 0.40	2.40 ± 0.10	1.44 ± 0.06
Muscle	1.74 ± 0.05	1.54 ± 0.08	1.72 ± 0.19	2.96 ± 0.43	1.12 ± 0.06	1.41 ± 0.27	1.23 ± 0.11
Skin	3.12 ± 0.13	2.02 ± 0.17	1.86 ± 0.22	2.94 ± 0.42	1.53 ± 0.07	1.86 ± 0.25	1.60 ± 0.16
Spleen	3.35 ± 0.26	2.64 ± 0.17	3.27 ± 0.37	9.42 ± 1.69	8.29 ± 0.56	15.49 ± 2.14	10.14 ± 1.46
	Recovery						
				% of injected dose			
	70.11 ± 6.64	35.73 ± 1.54	35.72 ± 4.11	56.58 ± 7.36	25.14 ± 0.95	27.94 ± 4.67	26.44 ± 2.39

Serum stability. After incubation of the oligonucleotide in mouse serum at 37° for 1 hr, very little full length oligonucleotide was present and the sample consisted of roughly equal amounts of oligonucleotides of reduced length and mononucleotides (Fig. 3a). Earlier time points (data not shown) indicated that half of the oligonucleotide was degraded in 10–30 min. After 2 hr only the monomer metabolites 5-methyl-2′-deoxycytidine and thymidine were found.

Tissue metabolism. Paired-ion HPLC analyses of nucleic acids isolated from tissue samples collected after injection of the MP-blocked phosphodiester oligonucleotide are shown in Fig. 3, b-d. Extremely rapid metabolism of the MP-blocked phosphodiester oligonucleotide was indicated by the fact that only the monomer metabolites could be found in organs taken from mice sacrificed 1 min after oligonucleotide injection.

Urine analysis. As can be seen in Fig. 3e, urine collected during the first 24 hr after MP-blocked phosphodiester oligonucleotide injection contained only the monomer.

Chimeric Oligonucleotide

Biodistribution. Results of the biodistribution studies with the tritiated chimeric oligonucleotide are shown in Table 3. In general, the uptake and clearance seem to be similar to those of the PS-blocked phosphodiester oligonucleotide (Table 1). As with the PS-blocked phosphodiester oligonucleotide, splenic accretion of radiolabel increased with time for the first 2 hr and remained relatively constant thereafter. The radiolabeled oligonucleotide was cleared rapidly from all other organs. The percentage of the injected dose remaining in the mice was less than that measured after administration of the MP-blocked phosphodiester oligonucleotide and equal to that obtained with the PS-blocked phosphodiester oligonucleotide.

Serum stability. The chimeric oligonucleotide demonstrated the greatest degree of serum stability (Fig. 4a) of the four oligonucleotides studied. The oligonucleotide appeared to be completely intact for the first 1 hr at 37°. After 5 hr, about 50% of the radioactivity was present in shortened oligonucleotides and mononucleotides (Fig. 4a). After 24 hr, the vast majority of the radiolabel was found in the monomer metabolites 5-methyl-2′-deoxycytidine and thymidine.

Tissue metabolism. Paired-ion HPLC analyses of the tissue content of kidney, liver, and spleen are shown in Fig. 4, b-d. The chimeric oligonucleotide was metabolized more slowly in these tissues than either the PS- or MP-blocked phosphodiester oligonucleotides. Intact oligonucleotide could be found in the kidney 20 min after injection, but all of the radioactivity was found in the terminal metabolites after 2 hr (Fig. 4b). Similarly, intact chimeric oligonucleotide was found in livers and spleens taken from mice 1 min after injection (Fig. 4, c and d).

Urine analysis. As can be seen in Fig. 4e, urine collected during the first 24 hr after injection of the chimeric oligonucleotide contained only the usual monomer metabolites.

Circular Phosphodiester

Biodistribution. Biodistribution of the tritiated circular oligonucleotide was unremarkable, compared with that of the unmodified or other blocked oligonucleotides (Table 4). Its clearance from the blood was rapid. Kidney uptake was initially very high, but the oligonucleotide was cleared with time. Accretion in the spleen increased with time, again suggesting uptake of metabolites. The overall pattern was essentially the same as observed previously for the phosphodiester oligonucleotide, indicating that the lack of 3′- and 5′-ends had no effect on biodistribution.

Serum stability. Despite the lack of a 3′-end, the circular oligonucleotide showed only a modest increase in stability in mouse serum (Fig. 5a). Mononucleotide metabolites were present within 30 min, but shortened oligonucleotide intermediates were not observed. The half-life of intact circle appeared to be about 40 min. After 2 hr, only mononucleotide metabolites were present.

Tissue metabolism. As shown in Fig. 5, b and c, tissue metabolism was rapid but was slightly slower for the circular oligonucleotide than for the unblocked and PS-blocked phosphodiester oligonucleotides. One minute after injection intact circular oligonucleotide was found in liver and kidney, and small amounts of intact circular oligonucleotide could be found in the kidneys 10 min after injection.

Urine analysis. As can be seen in Fig. 5d, urine collected during the first 24 hr after injection of the circular oligonucleotide contained only the usual mononucleotide metabolites.

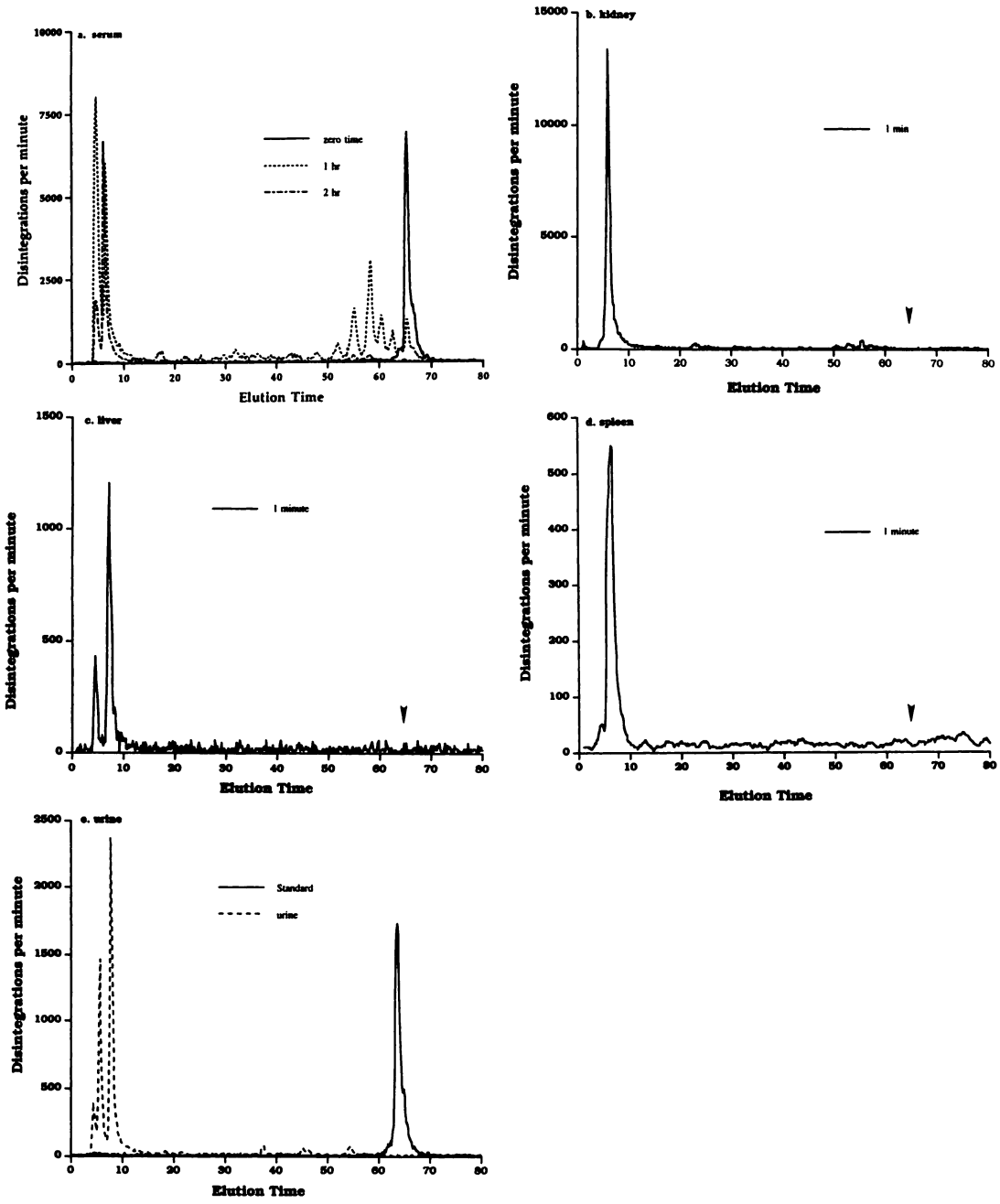

Fig. 3. Paired-ion HPLC of labeled nucleic acids present after addition or injection of the MP-blocked phosphodiester oligonucleotide. a, Serum (*in vitro*); b, kidney; c, liver; d, spleen; e, urine. *Arrowheads in b-d*, retention time of intact oligonucleotide.

Discussion

The pharmacokinetics and metabolism of two oligonucleotides (see Fig. 1) that differed only in the nature of their linkages were described in a previous report (18). The oligonucleotide with all PS linkages was relatively stable *in vivo* and showed properties making it suitable for use as a therapeutic agent, whereas an otherwise identical oligonucleotide with all phosphodiester linkages was degraded so rapidly that intact oligonucleotide could not be recovered from any tissues. The radiolabeling strategy used in that study, involving attachment of a tritiated methyl group at a single internal cytidine with *Hha*I methylase, ensured that radioactive metabolites were not released from the oligonucleotide until the oligonucleotide had been substantially degraded. The same labeling strategy and analytical techniques were used herein to study phosphodiester oligonucleotides with 3′- and 5′-ends protected from degradation by exonucleases. In the four oligonucleotides studied, the radiolabeled cytidine was surrounded by multiple phosphodiester linkages accessible to attack by endonucleases. Release of radiolabeled mononucleotides (5-methylcytidine and its metabolite thymidine) demonstrates that the phosphodiester center (but not necessarily the ends) of the oligonucleotide has suffered extensive degradation. Initially, the previously studied (18) phosphodiester oligonucleotide was protected on both ends with two PS linkages (PS blocked). The phosphodiesterase-

TABLE 3
Biodistribution of the tritiated chimeric oligonucleotide
Values are mean ± standard error (n = 5).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
				% of injected dose/g			
Blood	14.67 ± 1.98	4.81 ± 1.20	3.16 ± 0.24	1.55 ± 0.11	1.03 ± 0.06	0.81 ± 0.08	0.67 ± 0.09
Heart	3.83 ± 0.28	2.77 ± 0.13	1.59 ± 0.10	1.59 ± 0.17	1.15 ± 0.08	0.86 ± 0.08	0.45 ± 0.09
Lung	4.95 ± 0.16	3.72 ± 0.53	1.65 ± 0.42	1.55 ± 0.10	1.12 ± 0.07	0.96 ± 0.05	0.64 ± 0.17
Liver	4.74 ± 0.72	3.88 ± 0.66	2.50 ± 0.63	2.83 ± 0.11	2.20 ± 0.14	1.33 ± 0.10	0.53 ± 0.11
Kidney	8.15 ± 1.06	10.51 ± 2.63	10.06 ± 2.54	10.72 ± 0.70	4.40 ± 0.39	3.40 ± 0.46	0.49 ± 0.11
Muscle	0.83 ± 0.05	1.37 ± 0.01	1.03 ± 0.26	1.72 ± 0.13	1.16 ± 0.11	1.04 ± 0.10	0.43 ± 0.10
Skin	1.25 ± 0.21	2.53 ± 0.96	1.31 ± 0.38	2.28 ± 0.10	1.96 ± 0.19	1.73 ± 0.13	0.69 ± 0.16
Spleen	2.65 ± 0.26	3.42 ± 0.32	2.53 ± 0.65	4.25 ± 0.38	7.65 ± 1.14	9.58 ± 0.96	3.39 ± 0.48
	Recovery						
				% of injected dose			
	55.69 ± 2.71	40.03 ± 5.42	33.30 ± 7.13	45.88 ± 2.83	28.78 ± 2.05	25.17 ± 1.47	10.84 ± 1.28

resistant PS linkages were then replaced in a second oligonucleotide with phosphodiesterase-stable MP linkages (MP blocked). Interestingly, oligonucleotides blocked with MP linkages have been reported to be internalized somewhat more effectively than other blocked or unblocked oligonucleotides in cell culture studies (22, 23). Finally, both ends of the oligonucleotide were protected by joining the ends with a phosphodiester linkage, producing a circular oligonucleotide. Exonucleases obviously cannot be solely responsible for degradation of a circular oligonucleotide. Cyclization of oligonucleotides has been reported to render them completely (24) or partially (25) resistant to degradation in serum. The experiments that demonstrated complete stability in serum (24) were performed under unusual conditions (100 μM in human serum), which should produce dramatically longer apparent half-lives in serum than the conditions used herein (1 μM in mouse serum). Circular oligonucleotides are currently of interest because they can bind exceptionally tightly and selectively to some nucleic acid targets (26, 27) and can inhibit transcription factors (28). Because circular oligonucleotides similar in size to the other oligonucleotides used in this study are too small to possess these special properties, an unrelated circular oligonucleotide (designed to bind to the mRNA coding for the 170-kDa glycoprotein associated with multidrug resistance[2]) was tritiated and studied instead.

Because PS oligonucleotides are not rapidly cleared from mice, some type of serum binding and/or uptake presumably must be responsible for retention of these oligonucleotides (18). To determine whether the retention and relative stability of PS oligonucleotides *in vivo* might be partly explained by a binding or uptake process that is specific to oligonucleotides with many PS linkages, a "chimeric" 25-base oligonucleotide was designed with the radiolabel attached to a region with eight contiguous phosphodiester linkages. Fourteen PS linkages were placed on the 3′-side of this region to block 3′-exonucleases and to provide access to the postulated PS-specific binding and/or uptake. Because examples of oligonucleotide degradation by 5′-exonucleases were not known, the 5′-side of the phosphodiester region was minimally blocked with two PS linkages. The resulting chimeric oligonucleotide strongly resembled both the all-PS and PS-blocked oligonucleotides that had already been studied (Fig. 1b). Related chimeric antisense oligonucleotides with blocked ends and central regions capable of mediating mRNA cleavage by RNase H have been recommended by several groups (22, 29, 30). Human RNase H has been reported to prefer cleaving chimeric oligonucleotide/RNA duplexes with at least five contiguous phosphodiester linkages in the oligonucleotide (30). Studies with this chimeric oligonucleotide should establish whether a blocked oligonucleotide with a phosphodiester region acceptable to RNase H would be metabolically stable *in vivo*.

The data presented above clearly demonstrate that all of these strategies for blocking the ends of phosphodiester oligonucleotides did not provide adequate resistance to the nucleases present in mice. Except for the chimeric oligonucleotide, all of the phosphodiester nucleotides studied were found to be extensively degraded in tissues collected from mice sacrificed 1–10 min after injection. Even the chimeric oligonucleotide was significantly metabolized within 20 min. [In contrast, the PS oligonucleotide could be recovered partially intact from tissues up to 24 hr after injection (18).] As a consequence of this rapid degradation, the biodistribution of all four blocked phosphodiester oligonucleotides strongly resembled that of the unblocked phosphodiester oligonucleotide studied previously. Except for spleen and kidney, the concentrations of radiolabel in blood and tissues were approximately equal at all times later than 10 min after injection, consistent with a lack of uptake or retention in most tissues. Accumulation of radiolabel in spleen was observed with both blocked and unblocked phosphodiester oligonucleotides and was previously ascribed to uptake of labeled mononucleotide degradation products. For both blocked and unblocked phosphodiester oligonucleotides, the kidney/blood ratio remained greater than 1 for the first 1 hr and then decreased to about 1 in a manner previously characterized as being consistent with urinary excretion. The kidney/blood ratio remained significantly above 1 longer after injection of the chimeric oligonucleotide than after injection of the other phosphodiester oligonucleotides. Because PS oligonucleotides accumulate in the cortex of the kidney (18), the 14 PS linkages protecting the 3′-end of the chimeric oligonucleotide may have targeted this oligonucleotide to the kidney. The only labeled metabolites of blocked or unblocked phosphodiester oligonucleotides found in the urine

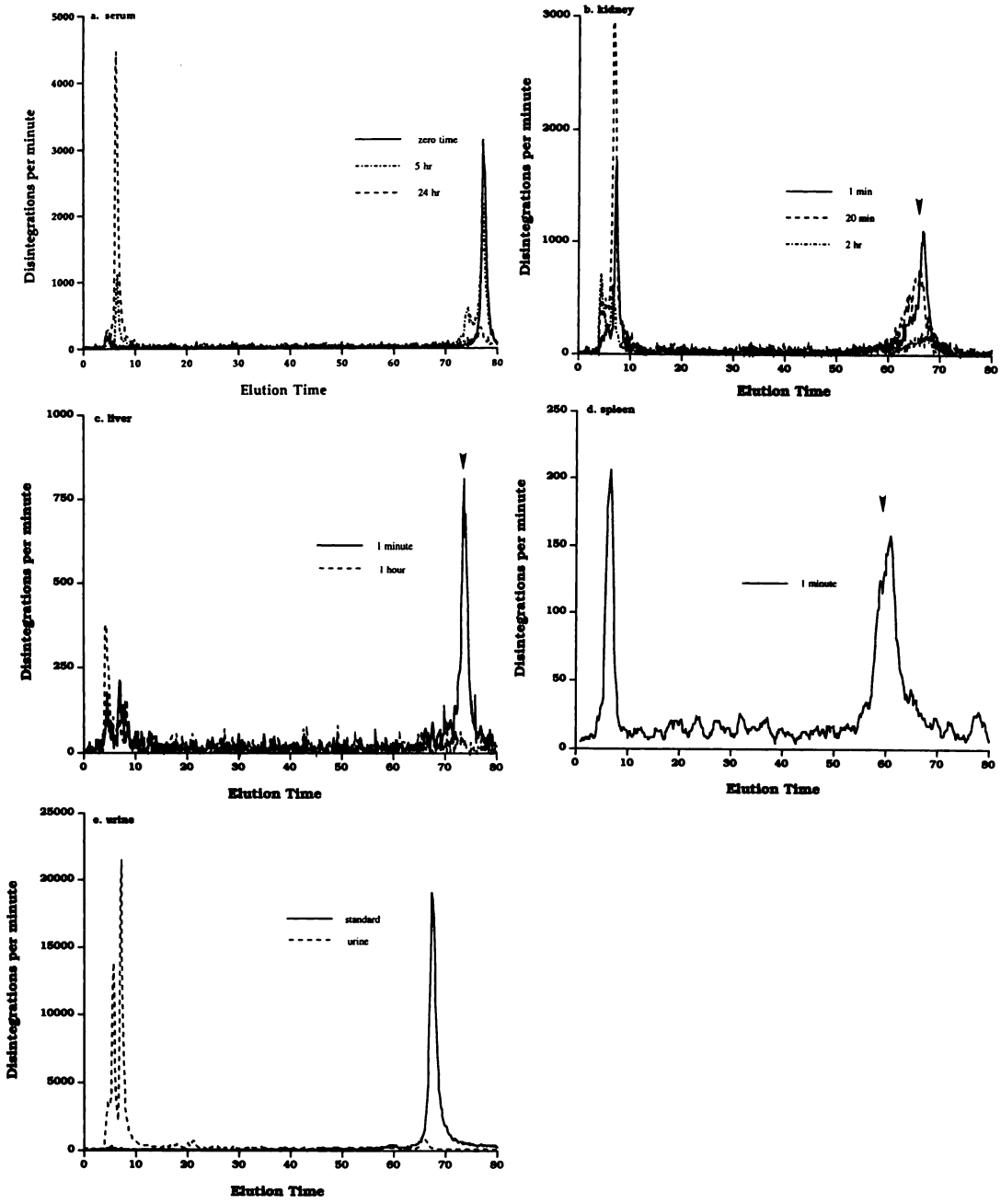

Fig. 4. Paired-ion HPLC of labeled nucleic acids present after addition or injection of the chimeric oligonucleotide. a, Serum (*in vitro*); b, kidney; c, liver; d, spleen; e, urine. *Arrowheads in b–d*, retention time of intact oligonucleotide.

were mononucleotides (whereas mono-, di-, and trinucleotide metabolites were found in urine after administration of labeled PS) (18). Because degradation was faster than renal clearance of intact oligonucleotide in all cases studied (possibly due to a high level of binding to serum proteins), renal clearance of an intact oligonucleotide was not observed.

Studies in mouse serum demonstrated that cyclization and blocking with PS and MP linkages provided partial, but not complete, protection against degradation. The apparent half-lives in serum for the PS-blocked, MP-blocked, and circular oligonucleotides were roughly 60, 20, and 60 min, respectively. Because different lots of mouse serum were eventually found to contain modestly different levels of nuclease activity, the differences between these half-lives were not judged to be significant. Under the same conditions, the parent unblocked phosphodiester oligonucleotide exhibited an apparent half-life, for loss of one base, of roughly 5–10 min (Fig. 6), so simple end-blocking clearly reduced degradation by exonucleases. [Under conditions where single-base resolution of degraded oligonucleotides was not achieved (18), the half-life of an unblocked phosphodiester in mouse serum was previously estimated to be 30 min. In that experiment, 30 min is now clearly seen to be the apparent half-life of a population of full length to nearly full length oligonucleotides capable of exerting potent antisense effects.] The chimeric oligonucleotide, with a half-life of about 5 hr, was clearly more stable in mouse serum than the other blocked oligonu-

TABLE 4

Biodistribution of the tritiated circular oligonucleotide

Values are mean ± standard error (n = 5).

Organ	Distribution						
	1 min	5 min	10 min	20 min	1 hr	2 hr	24 hr
	% of injected dose/g						
Blood	13.47 ± 3.22	1.71 ± 0.10	1.69 ± 0.37	1.99 ± 0.17	1.68 ± 0.16	1.53 ± 0.03	0.55 ± 0.03
Heart	4.69 ± 0.70	1.69 ± 0.14	2.14 ± 0.08	1.56 ± 0.09	1.96 ± 0.15	1.40 ± 0.06	0.49 ± 0.02
Lung	5.48 ± 1.15	1.59 ± 0.14	2.07 ± 0.12	1.50 ± 0.11	1.65 ± 0.12	1.49 ± 0.08	0.66 ± 0.05
Liver	4.15 ± 0.65	1.93 ± 0.16	3.82 ± 0.38	2.23 ± 0.19	2.41 ± 0.20	2.05 ± 0.13	1.17 ± 0.47
Kidney	15.04 ± 2.97	8.17 ± 0.75	8.92 ± 1.01	4.11 ± 0.37	2.88 ± 0.25	1.82 ± 0.06	1.30 ± 0.50
Muscle	1.03 ± 0.16	1.56 ± 0.14	2.50 ± 0.14	1.57 ± 0.18	1.74 ± 0.09	1.45 ± 0.08	0.50 ± 0.02
Skin	1.14 ± 0.30	1.49 ± 0.19	2.05 ± 0.15	1.93 ± 0.20	1.91 ± 0.20	1.74 ± 0.12	0.87 ± 0.03
Spleen	3.05 ± 0.69	2.51 ± 0.25	4.56 ± 0.31	5.57 ± 0.40	6.12 ± 1.86	15.60 ± 2.15	4.22 ± 0.67
	Recovery						
	% of injected dose						
	43.36 ± 6.64	30.41 ± 2.57	41.54 ± 1.90	28.18 ± 2.11	29.05 ± 1.89	24.44 ± 1.25	10.75 ± 1.06

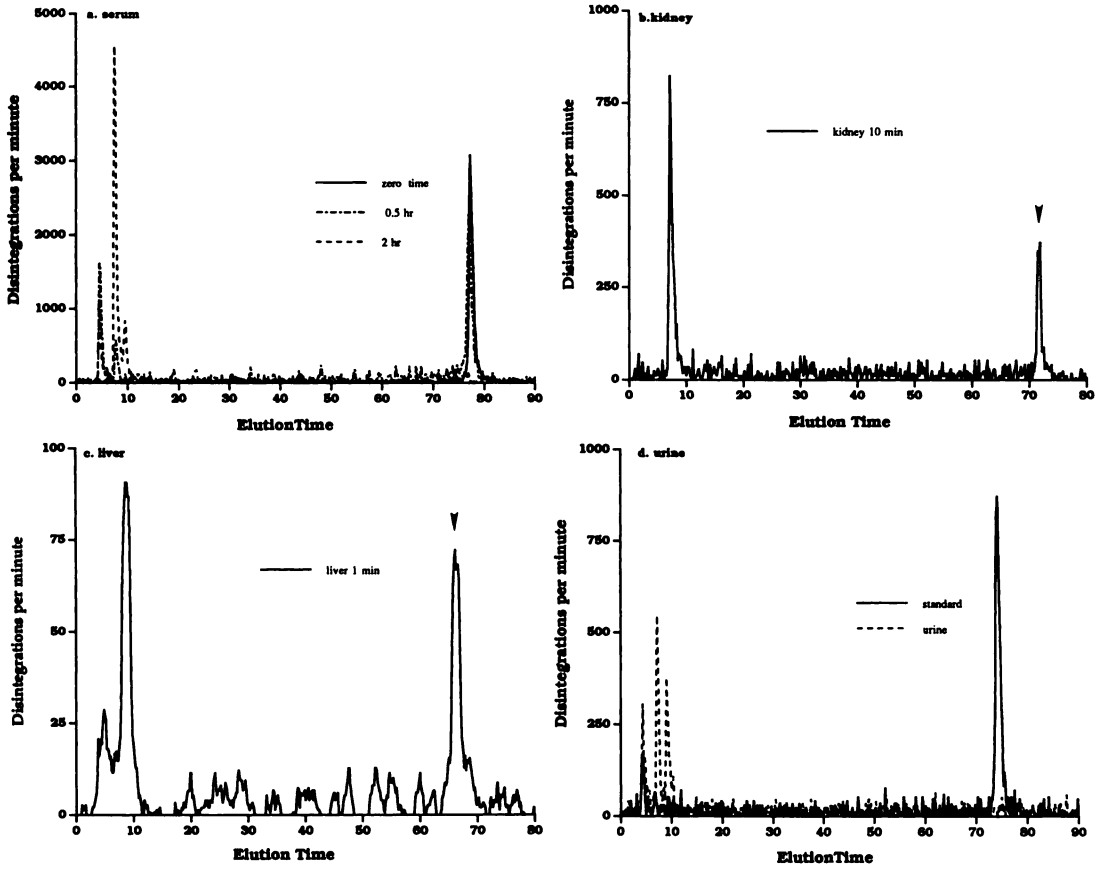

Fig. 5. Paired-ion HPLC of labeled nucleic acids present after addition or injection of the circular phosphodiester oligonucleotide. a, Serum (*in vitro*); b, kidney; c, liver; d, urine. *Arrowheads in b and c*, retention time of intact oligonucleotide.

cleotides. This increased stability (both in serum and *in vivo*) can be attributed to the fact that the chimeric oligonucleotide has fewer phosphodiester linkages available for attack.

These results clearly demonstrate that mouse serum contains endonuclease activity capable of initiating the degradation of blocked oligonucleotides over a period of about 1 hr in mouse serum at 37°. The simultaneous presence of both full length oligonucleotide and mononucleotide metabolites (see Fig. 4a, for example) implies that the fragments produced by endonucleases are rapidly degraded by exonucleases. Indeed, the circular oligonucleotide liberated labeled mononucleotide without detectable accumulation of any intermediates. These changes in degradation intermediates provide independent evidence that the blocking strategies have successfully inhibited the fastest pathway (exonuclease) in the degradation of unblocked phosphodiester oligonucleotides. Unlike analysis of changes in half-lives in serum, evidence of this type is not limited by uncertainty in estimating

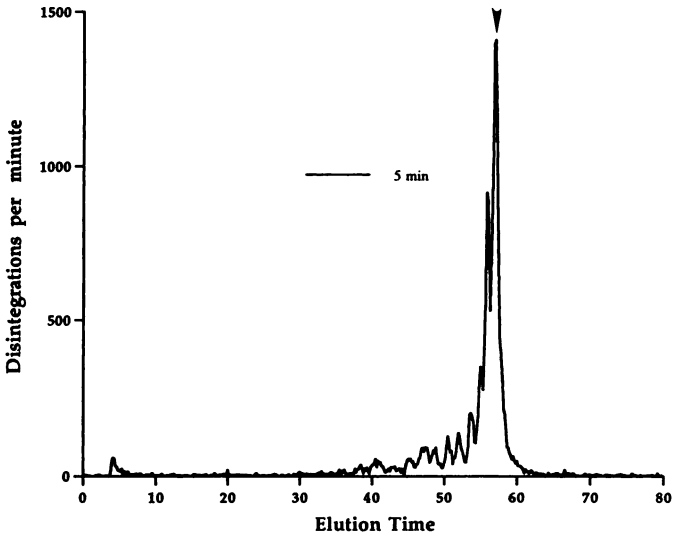

Fig. 6. Paired-ion HPLC of labeled nucleic acids present after incubation of the unblocked phosphodiester oligonucleotide in mouse serum for 5 min. *Arrowhead*, retention time of intact oligonucleotide.

half-lives in serum or by possible variations in serum nuclease activity.

The results of these stability experiments are generally consistent with previous studies (11–13) indicating that 3′-exonucleases are the major degradative enzymes in various sera. However, the level of endonuclease activity *in vivo* in mice and in mouse serum is high enough that oligonucleotide stability is only modestly increased by blocking strategies. It was not surprising to find that phosphodiester linkages are degraded more rapidly in serum and *in vivo* than in the dilute, heat-inactivated sera used in cell culture antisense experiments. However, the magnitude of the increase in the degradation problem was surprising. Blocking strategies that have previously afforded almost complete stability in cell culture experiments were only modestly successful in mouse serum and essentially worthless *in vivo*.

Our conclusion concerning the *in vivo* utility of blocked oligonucleotides differs from the conclusion reached by Zendegui *et al.* (17) using a guanine-rich, 38-base, phosphodiester oligonucleotide blocked on the 3′-end with a phosphopropyl amine. This blocked oligonucleotide exhibited uptake, clearance, and tissue distribution similar to those of all of our phosphodiesters, blocked or unblocked. In contrast to the results reported herein, however, intact 38-mer was recovered from tissues up to 8 hr after injection. Because *in vivo* degradation of our circular and chimeric oligonucleotides was clearly initiated by endonucleases, the stability of this 38-mer cannot be simply attributed to the 3′-phosphopropyl amine blocking group. This difference in stability could have several other explanations. First, the long, guanine-rich oligonucleotide used by Zendegui *et al.* (17) may have self-associated to form tetramers or aggregates (31) that degrade more slowly than monomeric oligonucleotides. Second, the post-labeling methodology used by Zendegui *et al.* could not detect terminal mononucleotide metabolites. Although other controls were used, those authors may have been misled by the low levels of detectable metabolites generated when slow endonuclease cleavage is followed by rapid exonuclease cleavage to mononucleotides. Finally, and most critically, Zendegui *et al.* used an oligonucleotide dose of 130 mg/kg, i.e., 20 times our dose. This difference alone can account for at least 1 order of magnitude change in apparent degradation rate. Given the expected high cost of manufacture of oligonucleotides, our dose of 6 mg/kg should provide a more realistic assessment of oligonucleotide stability. Thus, whereas the aforementioned experimental differences may account for some or all of the increased stability, the experiments described herein should be more relevant to potential therapeutic use of blocked antisense oligonucleotides.

In conclusion, our studies demonstrate that endonucleases present in mice are likely to rapidly inactivate any simple, monomeric, blocked phosphodiester oligonucleotide administered at practical doses. This conclusion probably also applies to chimeric oligonucleotides designed to mediate cleavage by RNase H, but our data do not rule out the remote possibility that marginal stability might be obtained when fewer than eight phosphodiester linkages are present (and possibly protected by adjacent nonionic linkages). It should be noted that oligonucleotide stability can vary with species; the circular oligonucleotide used in this study was significantly more stable in human serum than in mouse serum,[2] and reduced degradation of other oligonucleotides in human serum has been reported by others (9). It is therefore still possible that blocked or chimeric oligonucleotides could be stable enough in other species to permit therapeutic effects to be observed. The satisfactory pharmacokinetics and metabolism exhibited by PS oligonucleotides (18) make pursuit of blocking strategies in other species comparatively unattractive.

Acknowledgments

The authors would like to thank Dennis Watson, Timothy Hancock, and Ashley Beasley for their assistance with biodistribution and serum stability studies, Cheral Canna and Jerry Yarem for their assistance with radiolabeling, and George Trainor for his guidance.

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