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Real-Time Monitoring of the Hybridization Reaction: Application to the Quantification of Oligonucleotides in Biological Samples

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We describe here a competitive hybridization assay using TRACE technology which can be used for real-time monitoring of oligonucleotide hybridization. This assay quantifies all kinds of oligonucleotides in biological fluids without extraction. The assay makes use of two different probes and involves a fluorescent transfer process. As fluorescence measurements are not destructive, they can be sequentially repeated, thereby allowing comparison of the hybridization kinetics and binding strength of chemically modified backbone oligonucleotides (>0.5 nM) in biological media. The assay was validated for pharmacokinetic analysis of phosphodiester and phosphorothioate oligonucleotides in plasma and in different organs (liver, kidneys, lungs, spleen) at low concentrations (0.4 mg/kg, corresponding to clinical doses). Respective sensitivities for phosphodiester and phosphorothioate were 0.2 and 0.8 pmol/ml in plasma and 2 and 8 pmol/g in tissues, which allow to recover intact phosphorothioate sequences in some organs even after 24 h.

Key Words: **pharmacokinetic; phosphorothioate; oligonucleotides; hybridization; monitoring.**

The hybridization properties of nucleic acids have been widely exploited in developing methods of detection. The first nucleic acid detection methods involved hybridization on a solid support, as Dot Blot or Southern Blot [1, 2]. Currently, the most sophisticated method is DNA chips using hundred and sometimes millions of nucleic acid probes for detection of amplified DNA sequences. However, these methods are usually qualitative and not quantitative. Quantification based on hybridization is complicated since many factors can affect the reaction rate, such as intrinsic factors like length, GC ratio, the nature of the backbone of probes, and extrinsic factors like the presence of proteins, salt concentration. PCR is widely used for sensitive detection and quantification of RNA and DNA fragments. However, PCR is not applicable to oligonucleotides due to their small length (around 20 bases). Recently, we described a quantitative hybridization assay suitable for monitoring the plasma pharmacokinetics of low-concentration (<5 mg/kg) unlabeled antisense oligonucleotides with various backbones like phosphodiester, phosphorothioate and 2′-*O*-methyl (3; 4). This is a heterogeneous assay involving a solid-phase-immobilized sense-probe in which detection is achieved through the use of an avidin–acetylcholinesterase conjugate. The limit of quantification was below that of capillary electrophoresis, a reference method in this area [5–7]. Nevertheless, like all heterogeneous methods, unhybridized and hybridized sequences are separated by washing, which stops hybridization and lowers precision and sensitivity. Furthermore, it is not possible to follow the oligonucleotide's hybridization kinetic nor the strength of the binding, both of which are useful in improving the assay sensitivity. To overcome this problem, we decided to develop a homogeneous competitive hybridization assay (Fig. 1) using TRACE technology (Time Resolved Amplified Cryptate Emission) originally developed by Cis-Bio International [8–11]. This technique makes use of two different fluorescent probes (europium cryptate and allophycocyanin) and

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involves a fluorescence transfer process. It has already been used successfully in immunoassay [9, 11] and in DNA detection [8, 12] Here we describe the development of a homogeneous hybridization assay and its use in comparing the hybridization kinetics and affinity of different probes for a phosphodiester target backbone in biological media (plasma and organs extract), and the pharmacokinetic profiles of phosphodiester and phosphorothioate oligonucleotides.

MATERIALS AND METHODS

Reagents. All oligonucleotides were synthesized by Eurogentec (Seraing, Belgium) except for the PNA sequence which was synthesized by PE-Applied Biosystem (Cheshire, UK). Oligonucleotide sequences were antisense oligo A (5′-pTGAACACGCCATGTC-3′), antisense oligo ATS (phosphorothioate of oligo A throughout the sequence). Oligonucleotide reagents for competitive hybridization assay were sense target oligo B (5′-AAAAAAGACATGGCGTGTTCA-3′), sense target 2′-*O*-methyl (5′-AGTTGATCGGACGGGAAACG-3′) and sense target PNA (5′-GATCGGACGGGA-3′), and the competitor tracer C (5′-biotinTGAACACGCCATGTC-3′), CTS (phosphorothioate of oligo C throughout the sequence) and tracer C2 (complementary to PNA and 2′-*O*-methyl) (5′-biotinCGTTTCCCGTCCGATCA-ACT-3′).

The TBP-Eu^{3+} coupled to oligo B, 2′-*O*-methyl or PNA, and the acceptor, allophycocyanin, covalently bound to streptavidin (Str-APC) were kindly provided by Cis-Bio International. Blank mouse plasma and organs were collected from male Swiss mice (IFFA CREDO, l'Arbresle, France) and were stored frozen (−80°C). Aqueous hybridization buffer contained 0.75 M NaCl (Prolabo, Manchester, UK), 5 mM EDTA (Sigma, St. Louis, MO), 5 mM phosphate buffer pH 7 (Prolabo). Aqueous FIA buffer contained 90 mM Na_2HPO_4 (Prolabo, Manchester, UK), 27 mM KH_2PO_4 (Prolabo), 480 mM KF, 0.1% BSA (Sigma). Aqueous lysis buffer contained 20 mM Tris–HCl, pH 8 (Sigma), 20 mM EDTA (Sigma), 100 mM NaCl (Prolabo) and 0.5% Igepal (Sigma).

Apparatus. Assays were performed in microtiter plates (Micro-FLUOR "B," Dynatech Laboratories, France). Experiments were performed using a dedicated apparatus allowing double-wavelength detection (620 and 665 nm) (Cis-Bio International, Bagnols sur Ceze, France).

Labeling of sense oligonucleotides B at the 5′ end with TBP-Eu^{3+}. Two hundred and fifty microliters of 97 nM oligo B in distilled water was mixed with 200 μl of 0.1 M borate buffer pH 9. TBP-Eu^{3+} (360 nM) in acetonitrile was then added (three additions of 17 μl, one every 5 min) and the reaction product was purified by gel filtration on a Sephadex G50 fine column (NICK Spin column, Pharmacia Biotech, Uppsala, Sweden). The eluate containing the oligonucleotide B conjugate to TBP-Eu^{3+} at the 5′ end (oligo B-Kr) was analyzed by UV spectroscopy.

Real-time monitoring of hybridization. We first optimized the temperature of hybridization. The sense oligonucleotide B-Kr (labeled with TBP-Eu^{3+}) was diluted in FIA buffer at a concentration of 500 pM and the antisense oligonucleotide labeled with biotin (oligo C or CTS) was diluted in hybridization buffer at a concentration of 2 nM. Str-APC was added to each well at a concentration of 0.5 μg/well. The plates were covered with adhesive sheet and left at different temperatures. Every hour plates were placed in the Cis-Bio International apparatus and samples were excited by nitrogen laser pulse at 337 nm. The XL 665/TBP-Eu^{3+} fluorescence emission ratio was measured.

We also tested other backbones, 2′-*O*-methyl and PNA, 20 and 12 mer respectively, were labeled with TBP-Eu^{3+} at the 5′ end and diluted in FIA buffer. Complementary phosphodiester oligonucleotide (oligo C2) was biotinylated at the 5′ end and was diluted in CNTS human plasma. Str-APC was prepared as previously described.

Homogeneous competitive hybridization assay. The sense oligonucleotide B-Kr was diluted in FIA buffer at a concentration of 500 pM and the oligonucleotide tracer C or CTS was diluted in FIA buffer at a concentration of 2 nM. The assay was performed in a final volume of 250 μl (100 μl sense target, 50 μl tracer, 100 μl sample, standard, control sample or control extraction). The plates were covered with adhesive sheet and left overnight in an oven at the optimized temperature: 40°C for phosphodiester A and 35°C for phosphorothioate ATS. A 50-μl fraction of 10 μg/ml Str-APC was added to each well and the plates were left for one hour at room temperature before being placed in the Cis-Bio International apparatus. The samples were excited by nitrogen laser pulse at 337 nm, after which oligo B-Kr emits a characteristic long-lived fluorescence at 620 nm. A nonradiative energy transfer could occur between oligo-Kr (donor) and the second fluorescent label (acceptor, XL665 (Str-APC) fixed on oligo C or CTS via avidin-biotin interaction) only if an oligo B-oligo C (or oligo B-oligo CTS) duplex was formed. The ratio of XL665 emission (665 nm) to TBP-Eu^{3+} emission (620 nm) was measured and allowed real-time correction for optical variation of the biological matrix (plasma absorption, for instance). Immunofit EIA/RIA software (Beckman, France) with a four-parameter logistic transformation was used for calibration.

Validation procedure. Inter-assay accuracy, precision and limit of quantification were assessed using quality controls included in each assay and prepared as previously described [3].

The specificity of the competitive hybridization assay for phosphorothioate and phosphodiester oligonucleotides was determined as previously described [3].

In each organ, the extraction recovery of oligonucleotides was verified with three extraction controls which covered the range of the calibration curve. The concentrations of the extraction controls were determined for each assay and these data were accumulated over three assays. For each extraction control, recovery was calculated and for each organ an average extraction recovery was calculated.

Animal studies. Phosphodiester oligo A and phosphorothioate oligonucleotide ATS were injected (0.4 mg/kg) into male Swiss mice (30–35 g) via the tail vein. At the indicated times (5, 10, 15, 30 min, 1 h, 2 h, and 24 h), the mice were anesthetized with pentobarbital (60 mg/kg) and blood samples were collected by abdominal artery puncture. The organs (liver, lungs, spleen and kidneys) of three mice were removed at each time point. Blood samples were collected into heparinized tubes containing 10 μl of 500 mM EDTA to inhibit the nuclease activities. The plasma was obtained by centrifugation (2000*g*, 15 min, 4°C) and stored frozen (−80°C) until analysis. The organs were immediately frozen in liquid nitrogen and stored frozen (−80°C) until homogenization.

Extraction of oligonucleotide from tissues. Organs were homogenized in lysis buffer as previously described [13]: 1 ml of lysis buffer was added per 100 mg of tissues and homogenized in an ice bath. Homogenate (1 ml) was incubated with 2 mg of proteinase K at 37°C for 18 h. Each sample (500 μl) was extracted with 250 μl of phenol/chloroform (1/1) in a Phase Lock Gel tube (Phase Lock Gel Tube, TEBU, Le Perray en Yvelines, France). Samples were then centrifuged for 15 min at 12,000*g* at 4°C and the aqueous supernatant was removed and evaporated to dryness at 37°C under argon flow. The residue was resuspended in 500 μl of hybridization buffer containing 5 mM EDTA. These extracts were kept frozen (−20°C) until analysis.

Extraction controls were prepared to verify the extraction recovery by addition of different concentrations of oligonucleotide A or ATS to blank organs before homogenization.

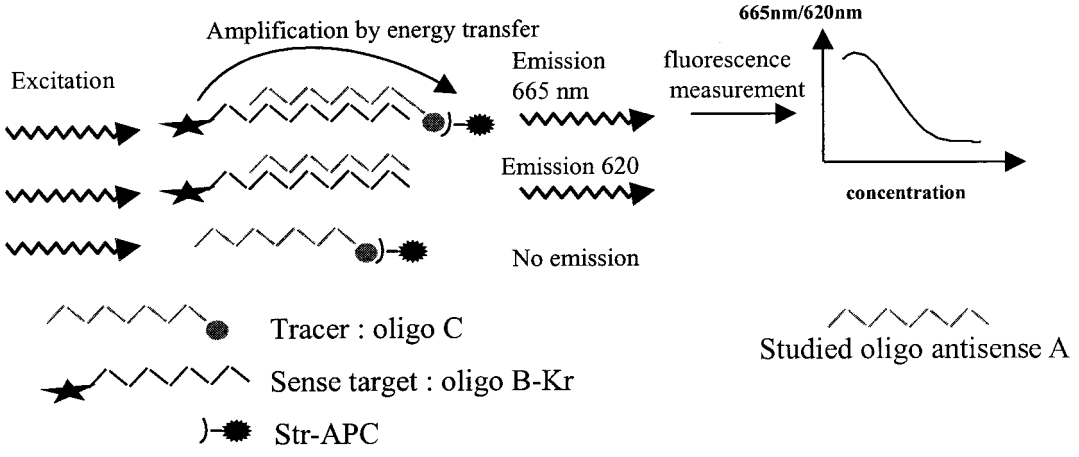

FIG. 1. Principle of the homogeneous competitive hybridization assay using TRACE technology. Sense target (oligo B) was labeled with TBP-Eu^{3+} (oligo B-Kr). Competitive hybridization occurs between tracer (oligo C) and antisense oligonucleotide (oligo A). The oligo C is labeled by a biotin molecule at the 5′ end so that it can bind a streptavidin–APC conjugate. The sense/tracer duplex (having bound Str-APC) is the only species in solution with the property of emitting at 665 nm after excitation at 337 nm due to a fluorescence transfer phenomenon.

RESULTS AND DISCUSSION

Homogeneous Hybridization Assay

This homogeneous assay (Fig. 1) is based on competitive hybridization between the antisense oligonucleotide (oligo A) and the tracer (oligo A with biotin at the 5′ end = oligo C) and the sense oligonucleotide (oligo B-Kr). The sense probe is labeled at the 5′ end with Eu^{3+} tribipyridine cryptate (TBP-Eu^{3+}, the donor). After excitation at 337 nm, the oligo B-Kr emits characteristic long-lived fluorescence at 620 nm. A nonradiative energy transfer can occur between oligo B-Kr (donor) and the second fluorescent label (acceptor, XL665 (Str-APC) fixed to oligo C via an avidin-biotin interaction) only if an oligo B-Kr-oligo C duplex is formed. The ratio of the fluorescence emission of XL665 (665 nm) to that of TBP-Eu^{3+} (620 nm) is measured and allows a real-time correction for optical variation of the biological matrix (plasma absorption, for instance). Moreover, as fluorescence measurements are not destructive they can be sequentially repeated without interrupting the hybridization process. As a consequence, this assay allows precise monitoring of hybridization kinetics and a precise quantification of all kind of oligonucleotides in biological fluids, by exploiting their hybridization properties.

Applications

Real-time monitoring of the hybridization reaction. Monitoring of the hybridization reaction allowed real-time comparison of the kinetics of hybridization of different probes, such as phosphodiester, phosphorothioate, PNA, 2′-*O*-methyl for a complementary phosphodiester sequence in human plasma or buffer, at various temperatures (Fig. 2).

In buffer, the initial rate of hybridization of the phosphodiester backbone increased with temperature from 30 to 40°C (Fig. 2A). This is due to Brownian motion which increases the probability of sense and antisense probes meeting. The signal plateaued 5 to 10 h after the beginning of the reaction. The plateau was reached faster at higher temperature. The plateau signal was higher at 35°C than at 30 or 40°C. The signal was the result of an equilibrium between association and dissociation of sense and antisense oligonucleotides. Tm is dependent on intrinsic parameters (GC ratio, . . .) and extrinsic parameters (salt concentration, protein concentration). Thus, 35°C should correspond to the experimental Tm of the sequence in buffer and under the homogeneous assay conditions. By contrast, in plasma at 40°C, the initial reaction rate was lower but the signal was higher than in buffer (Fig. 2B) and the experimental Tm was higher by about 5°C.

Kinetic profiles of phosphorothioate oligonucleotide backbone in buffer were also temperature-dependent (Fig. 2A). The initial rate was higher at 40°C than at 35 or 30°C. The plateau was reached 4 to 10 h after the beginning of the reaction. The maximum signal was observed at 30°C, which should correspond to the experimental Tm. These results are in accordance with those of Sauer *et al.* [14] which demonstrate that phosphorothioate oligonucleotides have a lower binding strength than phosphodiester oligonucleotides. In plasma, the hybridization kinetics are greatly modified since a 3-fold decrease in signal intensity was observed throughout (Fig. 2B). Furthermore, the plateau had not been reached 24 h after incubation. Phosphorothioate oligonucleotides bind avidly to proteins in plasma thus decreasing their affinity for the complementary target, as previously described [15].

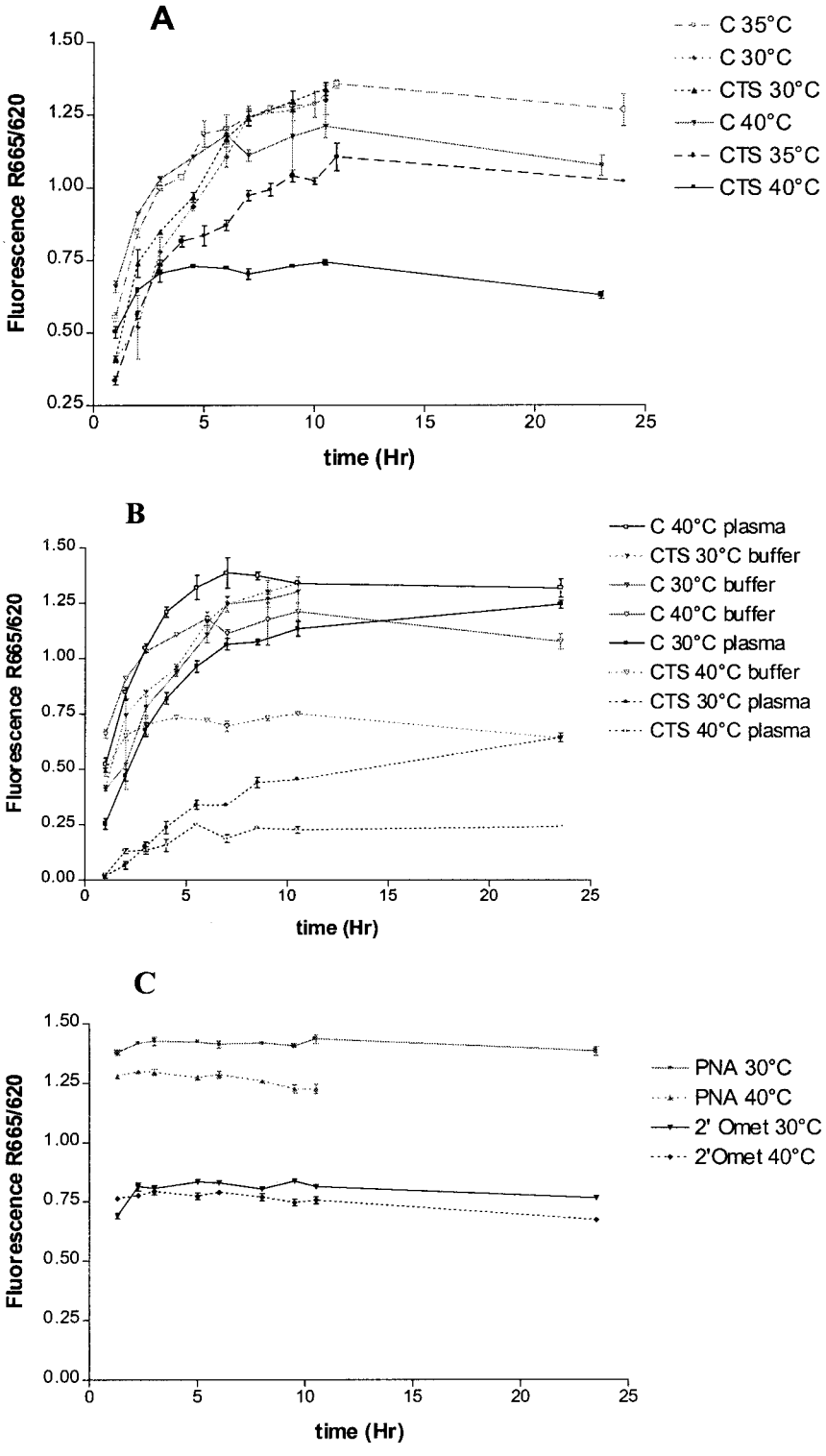

FIG. 2. (A) Kinetics of hybridization in buffer of a 15 mer phosphodiester antisense oligonucleotide C and a 15 mer phosphorothioate oligonucleotide CTS on their complementary sense target oligo B at different temperatures. (B) Comparison of the kinetic of hybridization in buffer and CNTS human plasma of phosphodiester oligo C and phosphorothioate oligo CTS on their complementary target oligo B. (C) Comparison of the kinetic of hybridization at different temperatures in CNTS human plasma of PNA and 2′-*O*-methyl oligonucleotide for their complementary phosphodiester target.

TABLE I

Accuracy (A%) and Precision between Days (CV%) of Antisense Oligonucleotide A and ATS in Plasma and Tissue Homogenate of Swiss Mice

CQ	Plasma		Liver		Spleen		Lungs		Kidneys	
(pmol/ml)	CV%	A%	CV%	A%	CV%	A%	CV%	A%	CV%	A%
					Oligo A					
0.2	15.3	*3.3*	24.2	*14.3*	22.6	*1.5*	16.5	*1.8*	13.2	*1.4*
0.5	15.8	*20*	14.5	*14.3*	15.7	*6.8*	12.5	*12*	8.7	*11.6*
1	1	*19*	16.6	*5*	17.1	*0.9*	8.2	*7.7*	14.5	*9.5*
2	11.1	*8*	18.3	*5.7*	10.9	*6.3*	12.6	*5*	13.5	*7.9*
					Oligo ATS					
1	12.9	*0.4*	11.37	*10*	1.8	*7*	12.4	*14.2*	20.1	*1.2*
2	19.2	*2*	13.4	*8*	17.4	*7.3*	4.2	*24*	9.8	*2.5*
5	7.8	*6.3*	11.4	*12.6*	5.5	*5.5*	16.3	*14.2*	14.5	*4.3*
25	12.5	*4.3*	2.7	*3.3*	16.6	*6.1*	2.3	*4.2*	15.3	*2.4*

Note. Each value is the mean of four assays.

Other modified oligonucleotides, such as PNA and 2′-*O*-methyl oligonucleotides, were tested in human plasma at 30 and 40°C (Fig. 2C). Signals observed with these 2 probes reached the equilibrium plateau very rapidly (1–3 h), in contrast to the phosphodiester and phosphorothioate backbones (5–10 h). These real-time monitoring data allowed us to optimize the competitive assays described below by adjusting the hybridization temperature. We believe this technique can be used to screen and compare the hybridization kinetics of oligonucleotides in various biological conditions, thus enhancing understanding of pharmacological activity *in vivo.*

Quantitative analysis of phosphodiester and phosphorothioate oligonucleotides. Antisense oligonucleotides with a phosphodiester or phosphorothioate backbone were quantified using a competitive homogeneous assay.

TABLE II

% of Cross Reactivity of Oligomers Deleted of 1 to 3 Bases at the 3′ and 5′ Ends

	Oligo A	Oligo ATS	
	40°C	35°C	30°C
3′ deletion			
N-1	58	56.5	100
N-2	24	22.5	100
N-3	<1	<1	ND
5′ deletion			
N-1	100	91.8	100
N-2	21	17	100
N-3	2.7	4	ND

Note. ND, not determined.

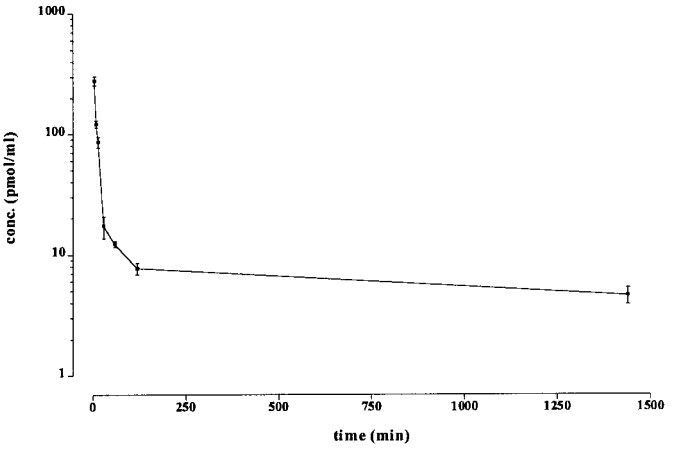

FIG. 3. Plasma concentration of intact phosphodiester oligo A and intact phosphorothioate oligo ATS after IV administration to mice at a single dose of 0.4 mg/kg. Each point is the mean of three determinations.

The hybridization temperature defined by real-time monitoring was fixed at 40°C for phosphodiester oligonucleotide, and at 35°C for phosphorothioate oligonucleotide [despite lower sensitivity than at 30°C since specificity was better (Table II)]. Validation parameters were assessed in mouse plasma and organ extracts (kidneys, lungs, liver and spleen) (Table I). Acceptable interassay CVs (<20%, 25% for lowest quality control concentration) were obtained in the calibration range: 0.2–2 nM and 1–25 nM for oligo A and ATS, respectively. The respective limits of quantification (loq) were 0.2 nM and 0.8 nM in plasma for phosphodiester oligo A and phosphorothioate oligo ATS, and 2 pmol/g and 8 pmol/g in tissues for oligo A and ATS. Our previously described heterogeneous method [3] was less sensitive (0.9 and 4 nM in plasma for oligo A and ATS, respectively), while specificity was similar (Table II). Enhanced sensitivity was clearly related to increased precision, very likely due to the absence of coating and washing steps. Moreover, the homogeneous assay is faster (readings can be made only half an hour after addition of Str-APC) and readings can be sequentially repeated without interrupting the hybridization process, until the equilibrium plateau is attained.

TABLE III

Extraction Recovery (%) of Oligo A and ATS in Tissues Homogenate of Mice

	Oligo A	Oligo ATS
Liver	90.4 ± 5.5	62.3 ± 2.3
Spleen	62.8 ± 2.4	24.8 ± 4.4
Lungs	74.3 ± 6.8	9.7 ± 0.8
Kidneys	63.7 ± 3.8	44.2 ± 4

Note. Each value is the mean of three assays at three concentrations.

In tissues, an extraction step was added. Extraction recovery (Table III) ranged from 60 to 100% for phosphodiester oligo A and 10 to 65% for phosphorothioate oligo ATS. These lower values for oligo ATS have already been reported [13] with this extraction technique. Phosphorothioates are more lipophilic than phosphodiesters so they bind more avidly to proteins [15] and were probably trapped in tissue debris and bound to proteins during the extraction process. But these extraction recovery values are below the 50% recommended by Shah *et al.* [16] for validating an analytical method. Nevertheless, except for spleen, these results are reproducible with an SEM below 10%. In the future, it would be better to develop another extraction procedure with better recovery of the phosphorothioate oligonucleotide. And present, pharmacokinetic data should be analyzed carefully in the case of phosphorothioate. Nevertheless, the whole extraction process can be controlled by adding another oligonucleotide sequence as internal standard to each sample before homogenization. The internal standard in the extract can be determined by another homogeneous assay to check stability and recovery for each sample.

This homogeneous assay gave a better limit of quantification than other specific methods such as HPLC [13, 17] and capillary electrophoresis [5, 18], where the quantification limit is around 10 nM in plasma and 60 pmol/g in tissues.

Pharmacokinetics. These techniques have been used to determine the plasma concentration-time profile of phosphodiester oligo A and phosphorothioate oligo ATS in mice following IV bolus administration of 0.4 mg/kg of oligonucleotide (Fig. 3). Five minutes after the injection, phosphodiester had completely disappeared from plasma whereas phosphorothioate was quantified until 24 h. It is important to note that phosphorothioate concentrations at 24 h were below the limit of quantification of other specific quantification techniques like capillary electrophoresis (10 nM). Table IV shows the pharmacokinetic parameters derived from a two-compartment analysis.

Figure 4 shows the tissue distribution. After injection of free phosphodiester, oligo A was not detected, except for a small amount in the liver. Phosphorothioate oligonucleotide accumulated most in kidneys and liver, with a maximum at 5 and 15 min, respectively.

TABLE IV

Plasma Pharmacokinetic Parameters Phosphorothioate Oligonucleotide ATS Following Intravenous Administration to Mice (Bicompartimental Analysis)

	AUC 0-inf (min*pmol/ml)	T1/2 a (min)	T1/2 b (min)	Vss (ml/kg)	Cl (ml/min/kg)
Oligo ATS	18672	4.6	812	4636	4.9

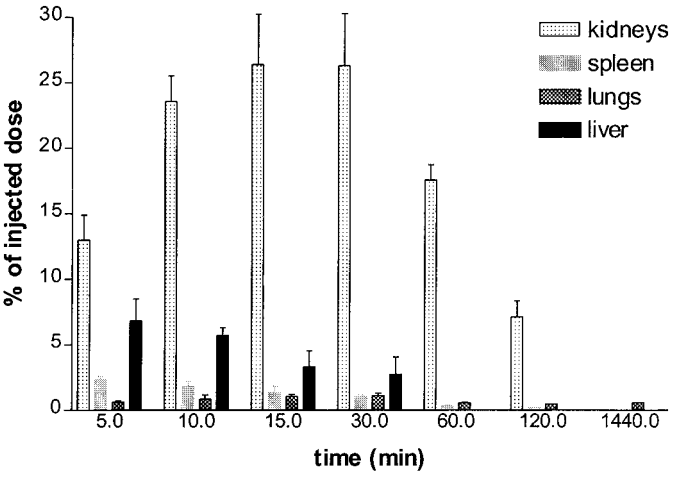

FIG. 4. Distribution in selected organs of intact phosphodiester oligo A and intact phosphorothioate oligo ATS after iv administration to mice at a single dose of 0.4 mg/kg.

Intact phosphorothioate was detected in the lungs 24 h after injection. These results, even if recoveries of phosphorothioate from organs are low, are in agreement with those of Crooke *et al.* [15] obtained using radioactively labeled compounds. These authors found high levels of total phosphorothioate in liver and kidneys up to 24 h. After metabolic analysis using HPLC combined with radioactivity detection, only 30 and 40% of intact phosphorothioate was seen in the kidneys and liver, respectively. Our method avoids labeling and thus can be performed in man. Our results, except for liver, are also in accordance with these obtained by Geary *et al.* [18] with a similar injected dose (0.8 mg/kg versus 0.4 mg/kg). Moreover, these authors confirmed the dose-dependent accumulation in tissues and so it is difficult to compare data when the injected doses differ.

Our work demonstrates that competitive homogeneous hybridization assay can be easily applied to monitor oligonucleotide hybridization in biological media containing proteins and to study oligonucleotide pharmacokinetics without the need for radiolabeling. The assay is more convenient than the heterogeneous assay, and can be optimized quickly. Only a few days are necessary to synthesize sense target and tracer oligonucleotide sequences and the assay could easily be developed further using an automated workstation.

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