Pharmacokinetics and Biodistribution of Oligonucleotide Adsorbed onto Poly(isobutylcyanoacrylate) Nanoparticles After Intravenous Administration in Mice

Yuichiro Nakada,[1,2] Elias Fattal,[2] Monique Foulquier,[2] and Patrick Couvreur[2,3]

Received June 2, 1995; accepted September 29, 1995

Purpose. The goal of this study was to evaluate the ability of nanoparticles to be used as a targeted delivery system for oligonucleotides.

Methods. Pharmacokinetic and tissue distribution were carried out in mice by measuring the radioactivity associated to the model oligothymidylate ^{33}P-pdT$_{16}$ loaded to poly(isobutylcyanoacryrate) (PIBCA) nanoparticles. In addition, we have used a TLC linear analyzer to measure quantitatively on a polyacrylamide gel electrophoresis, the amount of non degraded pdT$_{16}$

Results. Organ distribution study has shown that nanoparticles deliver ^{33}P-pdT$_{16}$ specifically to the liver reducing its distribution in the kidney and in the bone marrow. Nanoparticles could partially protect pdT$_{16}$ against degradation in the plasma and in the liver 5 min after administration, whereas free oligonucleotide was totally degraded at the same time.

Conclusions. Nanoparticles protect oligonucleotides *in vivo* against degradation and deliver them to the liver.

KEY WORDS: oligonucleotides; nanoparticles; pharmacokinetics; poly(isobutylcyanoacrylate); tissue distribution; stability.

INTRODUCTION

Antisense oligonucleotides have been used to inhibit the synthesis of cellular or viral proteins and might serve as potential therapeutic agents by the selective inhibition of gene expression (1). Therapeutic applications of antisense oligonucleotides have been severely hampered by their rapid cleavage by plasma nucleases and their very short half-life in the blood compartment (2, 3). In addition, these molecules poorly diffuse across the cell membrane, because of their ionic character. One strategy to improve the protection of oligonucleotides from the degradation and to increase their transport into the cells consists in their association with colloidal drug carriers (4, 5).

We have recently developed an original method allowing the efficient association of oligonucleotides with biodegradable poly(alkylcyanoacrylate) nanoparticles (5,6). This association was achieved by the formation of ion pairs between the negatively charged oligonucleotides and hydrophobic cations such as quaternary ammonium salts (5, 6). Schwab et al. (7) reported that when applied to anti-*ras* oligonucleotides, this delivery system markedly inhibited Ha-*ras*-dependent tumor growth in nude mice after intratumoral injection. Oligonucleotides bound to these nanoparticles were found to be protected from nucleases attack in cell culture media, and their cellular uptake was increased as a result of the capture of the nanoparticles by an endocytic/phagocytic pathway (6). However, the *in vivo* fate of oligonucleotides adsorbed onto these nanoparticles is not yet known although this parameter is determining for their therapeutic efficacy.

Thus the aim of this study was to evaluate the pharmacokinetic profile of the model oligothymidylate pd T$_{16}$ when associated with nanoparticles and administered intravenously to mice. The possibility to deliver this oligonucleotide to specific organs was also investigated.

MATERIALS AND METHODS

Materials

5'-phosphorylated oligothymidylate (pdT$_{16}$) was purchased from Pharmacia Biotech (St. Quentin Yvelines, France). The 5'-end labeled pdT$_{16}$ was synthesized using T4 polynucleotide kinase (Boehringer Mannheim, Germany) and ^{33}P-ATP (Amersham, France). Cethyltrimethylalkylammonium (CTAB) and isobutylcyanoacrylate monomer (IBCA) were purchased from Sigma (Saint Louis, USA).

Adsorption of Oligonucleotide onto Nanoparticles

Nanoparticles suspension was prepared by adding IBCA (10 mg/ml) to a solution containing HCl (pH 3) and dextran 70 (1%, w/v), in distilled water. The polymerization occurred spontaneously at room temperature under stirring for three hours. The mean size of the nanoparticles as measured by laser light-scattering (Nanosizer® ND4, Coultronics, Margency, France) was 233±27 nm (n=3). The nanoparticles suspension was then neutralized to pH 7 and diluted to a final concentration of 1 mg/ml, in 10 mM Tris-HCl buffer containing 1% (w/v) dextran 70. CTAB (final concentration, 300 μM) and ^{33}P-pdT$_{16}$ (final concentration, 1 μM) were added to the nanoparticles suspension in the presence of 150 mM NaCl. The suspension was incubated for two hours at room temperature. The adsorption percentage of ^{33}P-pdT$_{16}$ at the surface of the nanoparticles determined as described previously (5) was 94.4±2.5% (n=3).

In Vitro Stability of ^{33}P-pdT$_{16}$ in Plasma

Degradation experiments were carried out at 37°C in 70% of mouse fresh plasma. 50 μl of plasma samples were used for the determination of the amount of intact pdT$_{16}$. The concentration of ^{33}P-dT$_{16}$ (either free or nanoparticles-bound) was 1 μM in the incubation medium. The nanoparticles concentration was 1 mg/ml. Oligonucleotides were extracted from plasma once with 50 μl of phenol/chloroform/iso-amylalcohol (25:24:1, v/v/v) and once with 50 μl of chloroform/iso-amylalcohol (24:1, v/v) as described previ-

[1] Pharmaceuticals Research Center, Kanebo, LTD., 5-90, Tomobuchi-cho, 1-Chome Miyakojimaku, Osaka 534, Japan.

[2] Laboratoire de Physico-chimie-Pharmacotechnie-Biopharmacie, URA CNRS 1218, Université Paris-Sud, Faculté de Pharmacie, 92296 Chatenay-Malabry, Cedex, France.

[3] To whom correspondence should be addressed.

0724-8741/96/0100-0038$09.50/0

ously (8). After centrifugation (5, 900g, 10 min) of the plasma samples, the aqueous phase was directly used for the electrophoresis using a 20% polyacrylamide-7 M urea sequencing gel. Bands were analyzed quantitatively by measuring the radioactivity using an AUTOMATIC TLC-LINEAR ANALYZER® (Berthold, Germany). Results were treated in order to reduce the background of radioactivity.

Animal Experiments

Male OF1 mice (5 weeks) received injections into the tail vein of ^{33}P-pdT$_{16}$ free or adsorbed onto nanoparticles at a dose of 5 nmol/5 ml/Kg corresponding to a dose of polymer of 5 mg/Kg. The amount of radioactivity the animals received was from 1.6 to 5.2 μCi/Kg. Test animals for the pharmacokinetics were sacrificed at 2, 5, 15, 30, 60, 120, 240, 360 min and 24 hr after intravenous administration and at 5, 30, 120 min and 24 hr for organ distribution studies. Blood and organs were removed for the determination of the radioactivity by scintillation counting after digestion of the tissues with 1 ml of SOLUEN® 350.

In Vivo Stability of pdT$_{16}$ in the Plasma and in the Liver

Male OF1 mice (5 weeks) received injections of ^{33}P-pdT$_{16}$ free or adsorbed onto nanoparticles at a dose of 5 nmol/89-145 μCi/5 ml/Kg. They were sacrificed at 5 min after the administration. Then, blood and liver were removed and the extraction procedure performed as described (8). Practically, liver was homogenized with 5 times its weight of ice-cold water. Fifty μl of plasma samples and 300 μl of liver homogenates samples were adjusted to pH 12 with sodium hydroxyde to dissolve the nanoparticles and incubated for two hours at 37°C, as previously described (6). After incubation, samples were adjusted to pH 7 with hydrochloride acid. Plasma samples were treated as described above. Liver homogenate samples were treated with 30 μl of proteinase K (0.5 mg/ml) in extraction buffer (0.5% SDS, 10 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.6) for three hours at 37°C, then these liver samples were extracted once with 600 μl of phenol/chloroform/iso-amylalcohol (25:24:1, v:v:v) and once with 600 μl of chloroform/iso-amylalcohol (24:1, v:v). After centrifugation (5900g, 10 min), the aqueous phase was precipitated with ethanol and 3 M NaCl and kept one hour at -20°C. These precipitates were resuspended in 80 μl of distilled water. Samples were then analyzed by electrophoresis as described above. Concentrations of undegraded pdT$_{16}$, expressed in pmol/g or ml, were calculated according to the following equation:

$$pdT_{16}\text{intact(pmol/ml or g)} = \frac{A}{T}(\%).C$$

where A is the area under the peak corresponding to undegraded pdT$_{16}$, T being the total amount of radioactivity in the liver or plasma sample and C the total concentration of oligonucleotide (pdT$_{16}$ degraded plus undegraded) per g of liver or ml of plasma.

RESULTS

Plasma Concentration and Tissue Distribution

Plasma levels of radioactivity after intravenous administration of ^{33}P-pdT$_{16}$ free or ^{33}P-pdT$_{16}$ nanoparticles are shown in Fig. 1. Plasma concentration profiles and the resulting pharmacokinetic parameters calculated by a non-linear regression using a two-compartments model were very similar for both formulations (oligonucleotide free or associated with nanoparticles) (Fig. 1). The half-lives of ^{33}P-pdT$_{16}$ in the distribution phase after administration of ^{33}P-pdT$_{16}$ free or adsorbed onto nanoparticles were 7.1 and 10.2 min, respectively (Fig. 1). The total clearance and AUC$_{0-\infty}$ of ^{33}P-pdT$_{16}$ free were again very similar to those of ^{33}P-pdT$_{16}$ associated with nanoparticles. On the contrary, as shown in Fig 2, the concentration of the radioactivity in the organs was very different for ^{33}P-pdT$_{16}$ free and associated with nanoparticles. Liver concentration at 5 min after the administration of ^{33}P-pdT$_{16}$ nanoparticles was twice that of ^{33}P-pdT$_{16}$ free (Fig. 2). On the contrary, nanoparticles reduced the concentration of radioactivity in the kidneys and the bone marrow by about 4-times at 5 min (Fig. 2). At time 30 min, the reduction of radioactivity was still important for the bone marrow. At the same time, the concentration of the radioactivity in the spleen, in the lung and in the muscle was lower after the administration of ^{33}P-pdT$_{16}$ nanoparticles than for free ^{33}P-pdT$_{16}$. Brain concentrations were very low for both treatments.

In Vitro Stability of pdT$_{16}$ in Plasma

The degradation kinetic of pdT$_{16}$ was tested *in vitro* in 70% of mouse plasma at 37°C. The half-lives of pdT$_{16}$ incubated free and bound to nanoparticles were 6.0 and 12.5 min, respectively (Fig. 3). Thirty minutes after incubation in plasma, the percentage of unchanged pdT$_{16}$ was 2.9% for

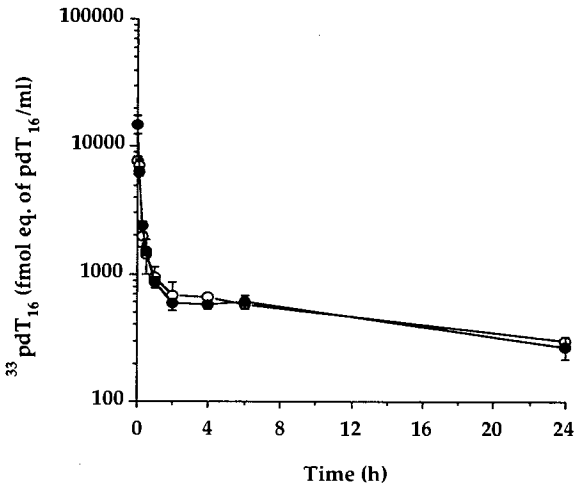

Fig. 1. Radioactivity levels in plasma after intravenous administration of ^{33}P-pdT$_{16}$ free (○) or ^{33}P-pdT$_{16}$ associated to PIBCA nanoparticles (●) at a dose of 5 nmol/Kg. Means ± S.D. of 3 to 4 mice. Pharmacokinetics parameters are fitted according to a two compartment model ($y = Ae^{-\alpha t} - Be^{-\beta t}$). α (1.h^{-1}) and β (1/h^{-1}) are fast and slow rate constants respectively. Clt (ml. h^{-1}.kg^{-1}) corresponds to the total clearance and AUC0$^{-\infty}$ (fmol.h.ml^{-1}) is the area under the curve. The values of α, β, Clt, and AUC0$^{-\infty}$ are 5.86±0.67, 0.046±0.006, 254 and 19692 respectively for nanoparticle-associated ^{33}P-pdT$_{16}$. These values are α = 6.78±0.8, β = 0.046±0.008, Clt = 260 and AUC0$^{-\infty}$ = 19268 for free ^{33}P-pdT$_{16}$.

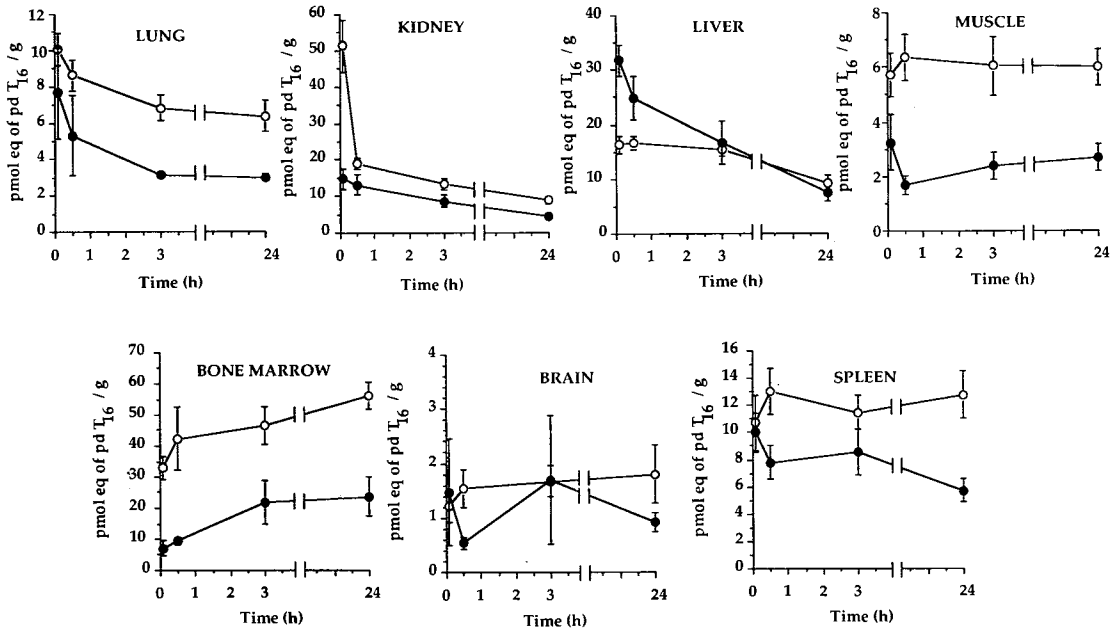

Fig. 2. Radioactivity in tissues after intravenous administration of ^{33}P-pdT$_{16}$ free (○) or ^{33}P-pdT$_{16}$ associated with PIBCA nanoparticles (●) at a dose of 5 nmol/Kg to male mice. Means ± S.D. of 3 to 5 mice.

free oligonucleotide whereas 28.9% of ^{33}P-pdT$_{16}$ associated with nanoparticles was still intact (Fig. 3).

In Vivo Intact pdT$_{16}$ in Plasma and Liver

Plasma and liver samples (taken 5 min after ^{33}P-pdT$_{16}$ administration) were analyzed for the determination of intact oligonucleotide. Electrophoresis chromatograms of plasma samples and liver homogenates are shown in Fig 4 and 5. Undegraded pdT$_{16}$ could be detected in the plasma and in the liver after the administration of the oligonucleotide associated with the nanoparticles, but not in the case of the oligonucleotide administered free. In the case of the administration of nanoparticles, the percentages of undegraded pdT$_{16}$ (as calculated from the area in the electrophoresis chromatograms) were 20.1% for the plasma and 61.2% for the liver. Therefore, the calculated intact pdT$_{16}$ concentrations were 1.27 pmol/ml in the plasma and 19.4 pmol/g in the liver.

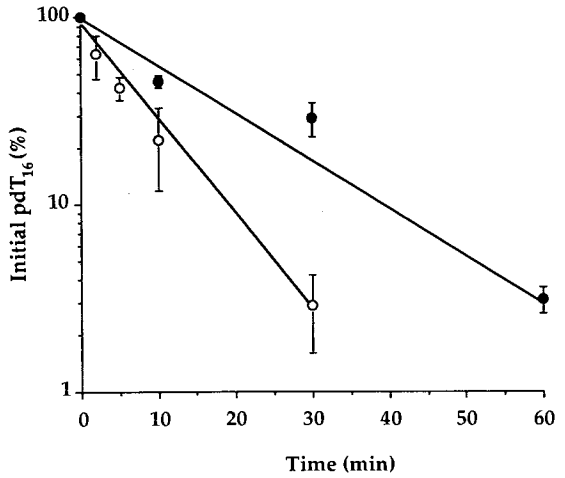

Fig. 3. Kinetics of degradation of free ^{33}P-pdT$_{16}$ 1 μM (○) or ^{33}P-pdT$_{16}$ associated to PIBCA nanoparticles 1 μM (●) in 70% mouse plasma. Means ± S.D. (n=3).

DISCUSSION

In a recent report, we have described an efficient method for adsorbing the oligonucleotide pdT$_{16}$ at the surface of poly(alkylcyanoacrylate) nanoparticles (5). These pdT$_{16}$ nanoparticles exhibited a better stability towards phosphodiesterases enzymes and an increased cellular uptake (6). As a preliminary *in vivo* evaluation of this system, we now focused on the behavior of pdT$_{16}$ nanoparticles after intravenous administration. Pharmacokinetics and tissue distribution of oligonucleotides free after intravenous administration were studied by others (3, 9, 10, 11, 12). These studies demonstrated that oligonucleotides behave similarly to other macromolecular agents in regards to renal elimination. The kidney possesses the highest initial amount of radio label suggesting that this organ may play an important role in the elimination process.

The present results have shown that although nanoparticles did not markedly increase the blood half-life of the ^{33}P-pdT$_{16}$, its tissue distribution was significantly modified. Indeed, after the administration of ^{33}P-pdT$_{16}$ associated to nanoparticles, a strong liver uptake was observed associated to a subsequent reduced distribution in the other organs, especially in the kidney. These data suggest that with the aid of nanoparticles, pdT$_{16}$ could be delivered to the liver with a certain specificity. This is clearly illustrated by the calculated percentage of the liver radioactivity over all the other organs: this value of 14.5% for pdT$_{16}$ free was dramatically increased to 71.5% with nanoparticles (time 5 minutes). It is generally accepted that, after intravenous administration, submicronic polymeric particles are taken up by the mononuclear phagocyte system, particularly by the Kupffer cells

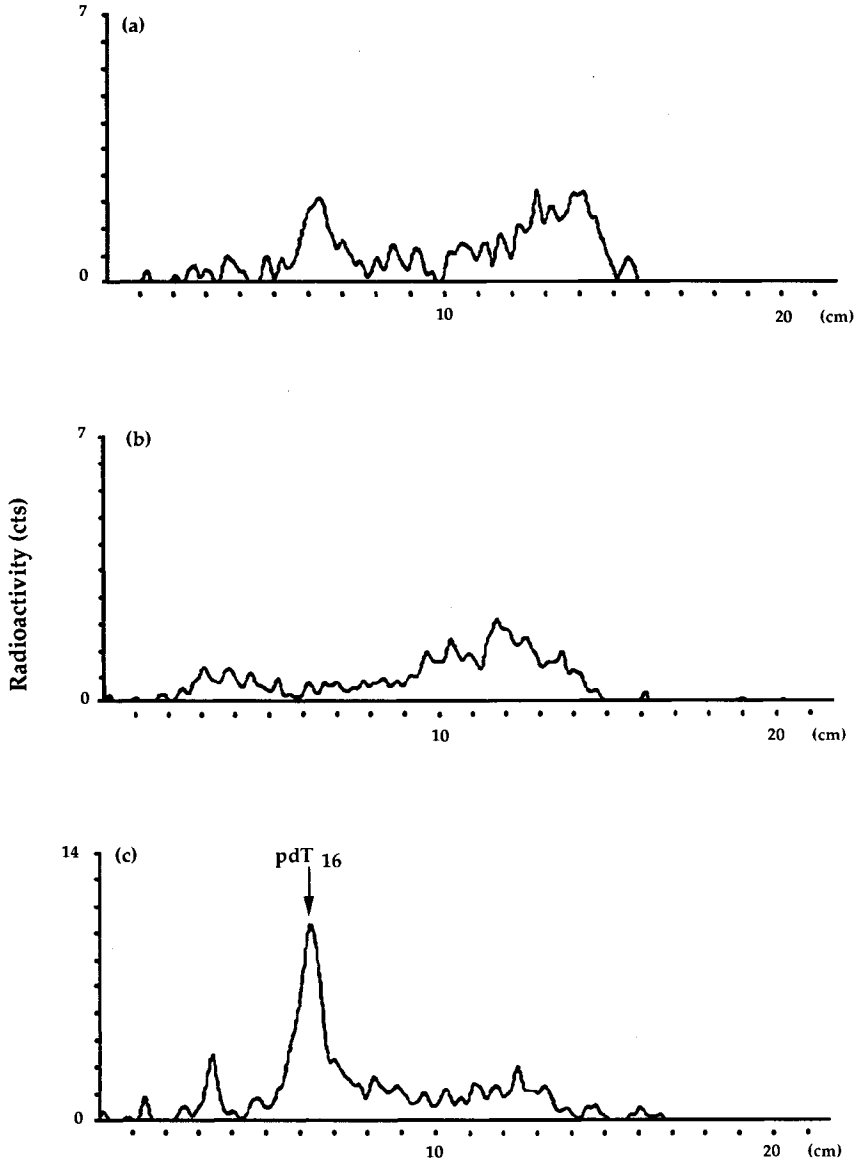

Fig. 4. Electrophoresis chromatograms of intact pdT_{16} in plasma 5 minutes after intravenous administration (5 nmol/Kg) of ^{33}P-pdT_{16}.

(13). This explains the important liver uptake of the nanoparticle-bound pdT_{16}. To address the crucial problem of oligonucleotide degradation, we have investigated the state in which (degraded or not) oligothymidylate was in the plasma and delivered to the liver at 5 minutes after intravenous administration. It should be pointed out that the evaluation of the *in vivo* stability of oligonucleotides represents a major technical difficulty. Two techniques are generally utilized to quantify this degradation: autoradiography (3) and HPLC (9). None of these methodologies are able to combine a high sensitivity with an accurate quantitative determination of the undegraded oligonucleotide. In this paper, an original method is proposed that consists in the use of a TLC analyzer allowing, in polyacrylamide gels, to quantify the amount of undegraded pd T_{16} in tissues such as liver and plasma (14). Using that methodology, it was found that a significant amount of ^{33}P-pdT_{16} was kept intact in the liver and the plasma when administered under the form of nanoparticles. When injected free, no traces of ^{33}P-pdT_{16} were detected in the same tissues.

Although PIBCA nanoparticles showed interesting potentialities for liver targeting, its efficacy in terms of protection of oligonucleotides from the degradation should be improved. In fact, in the stability experiment that we carried out *in vitro*, some degradation of the adsorbed oligonucleotide occurred in the presence of plasma. This could be attributed to the leakage of pdT_{16} from the nanoparticle solid phase. Indeed, since oligonucleotides molecules were adsorbed onto the particle surface through the formation of ion pairs, it is assumed that a fast release could occur as a consequence of a competition with the plasma proteins. This may probably explain why the oligonucleotides became partly degraded even when adsorbed onto nanoparticles. Another explanation might be provided by the surface bio-

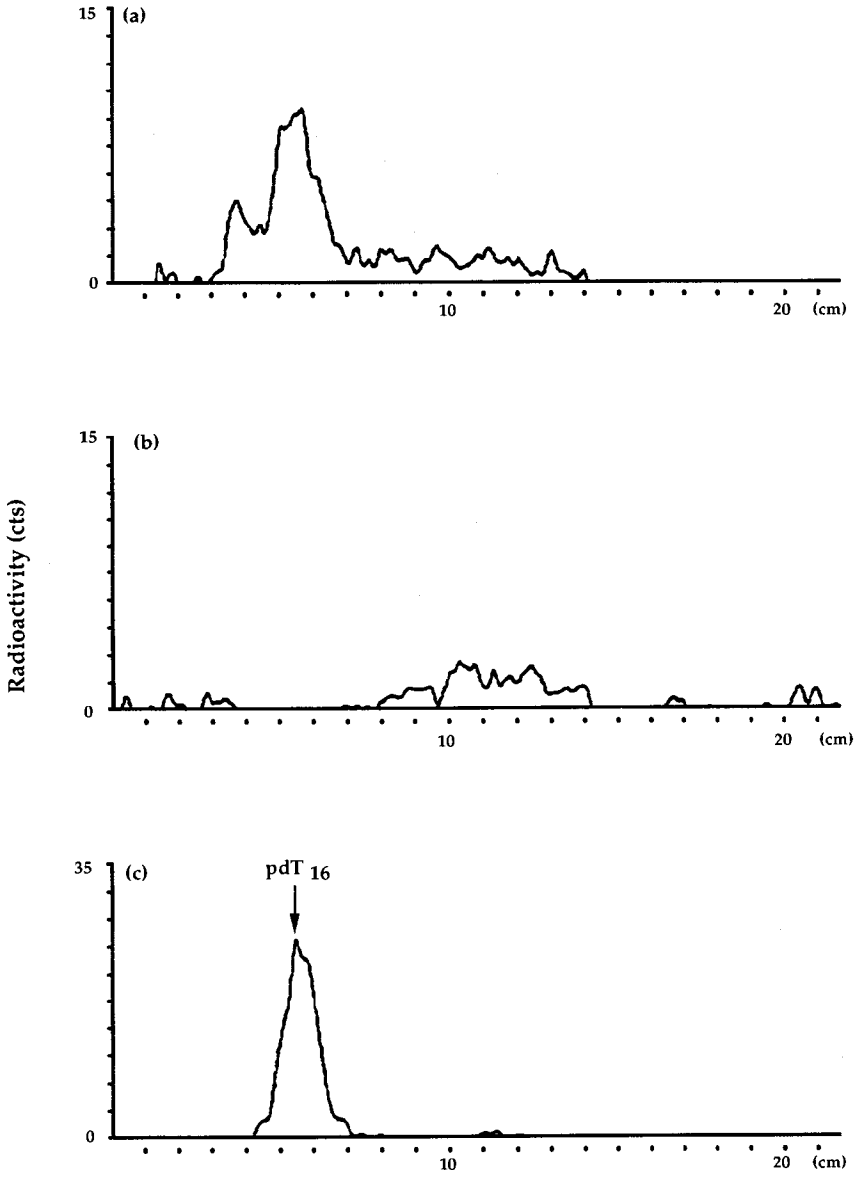

Fig. 5. Electrophoresis chromatograms of intact pdT_{16} in liver 5 minutes after intravenous administration (5 nmol/Kg) of ^{33}P-pdT_{16} associated to PIBCA nanoparticles (a) or of $^{33}pdT_{16}$ free (b). (c) is the control (liver homogenate spiked with 18,000 dpm of intact $^{33}pdT_{16}$).

erosion of the nanoparticles, due to serum esterases (15) and subsequent quick desorption of the oligonucleotide molecules. One suggestion would be to develop a nanoparticulate system that would entrap the oligonucleotide molecules and retains them more efficiently inside the polymer network. Nevertheless, our data show that only a coating was already efficient in inducing the oligonucleotides to reach the liver and to be partly protected in this tissue against degradation.

In conclusion, this study has shown that the organ distribution profile of ^{33}P-pdT_{16} could be dramatically modified after association with PIBCA nanoparticles with an increase of liver concentration while the kidney concentrations are reduced. These results suggest that nanoparticles might be considered as an interesting carrier system for the treatment of liver diseases such as viral hepatitis or liver cancers and metastasis.

ACKNOWLEDGMENTS

This paper was supported by ARC (Association pour la Recherde sur le Cancer) grant No. 1188.

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