Journal of Chromatography, 533 (1990) 133–140
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam

CHROMBIO 5502

High-performance liquid chromatographic analysis of phosphorothioate analogues of oligodeoxynucleotides in biological fluids

JAMES C BIGELOW, LYNN R CHRIN, LINDA A MATHEWS and JOHN J McCORMACK*

Department of Pharmacology, University of Vermont, Burlington, VT 05405 (U S A)

(First received April 3rd, 1990, revised manuscript received July 24th, 1990)

ABSTRACT

Phosphorothioate oligodeoxynucleotides (S-ODNs) have potential as anti-viral agents and are being investigated for the chemotherapy of AIDS A high-performance liquid chromatographic method is described for the analysis, in urine and plasma, of a 28-unit deoxycytidine homopolymer (S-dC_{28}) and a 28-unit S-ODN "antisense" to the *rev* gene of the human immunodeficiency virus This method employs ion-pairing HPLC with a polymeric column Tetrabutylammonium is used as the ion-pairing agent in a mobile phase of acetonitrile in pH 7.0 phosphate buffer Analysis of the S-ODNs is relatively rapid (20 min) and sensitive (20 nm) and is accomplished by a gradient elution (22.5–30 0% acetonitrile) followed by ultraviolet (266 or 272 nm) absorption detection This method is likely applicable, with appropriate modifications, to all S-ODNs of similar molecular weight regardless of sequence The S-ODNs bind very strongly to plasma proteins but are readily prepared for analysis by a phenol extraction procedure. In a preliminary pharmacokinetic study in mice with S-dC_{28}, very rapid elimination of the oligomer from plasma was observed (half-time, 11 6 min) Estimates for the apparent volume of distribution and total body clearance were 3 ml and 0 2 ml/min, respectively It appears that the majority of the oligomer is eliminated by renal clearance (glomerular filtration), a property likely shared by all S-ODNs of similar molecular mass

INTRODUCTION

Oligodeoxynucleotides (ODNs) synthesized to be complementary or "antisense" to specific RNA sequences have been demonstrated to have great potential as chemotherapeutic agents [1,2]. Of special interest are phosphorothioate analogues (S-ODNs) in which one non-bridging oxygen atom has been replaced by a sulfur atom in each internucleotide phosphate linkage. These S-ODNs are resistant to nuclease hydrolysis [3], form water-soluble salts and are excellent candidates as chemotherapeutic ODNs.

Matsukura *et al.*[4] have reported that a 28-unit deoxycytidine homopolymer S-ODN (S-dC_{28}; NSC 613671) is a potent inhibitor of the replication of the human immunodeficiency virus (HIV) *in vitro*. Antisense S-ODNs have also been reported to show high activity against HIV *in vitro* [5]. A 28-unit S-ODN (5′-TCG TCG CTG TCT CCG CTT CTT CCT GCC A-3′; NSC 613672) antisense to a

0378-4347/90/$03 50

region of the *rev* gene of the HIV has been selected for possible development as an anti-AIDS agent. This S-ODN was originally designated as "S-ODN-4" by Stein *et al.*[6] and is designated herein as such for convenience of presentation.

Use of these two agents and of S-ODNs in general in chemotherapy will require pharmacokinetic and related studies. However, there is currently no method reported to measure the concentration of S-ODNs in biological fluids. This paper describes a sensitive (20 n*M*) and relatively fast (20 min) high-performance liquid chromatographic (HPLC) method for analysis of S-dC_{28} and S-ODN-4 in plasma and urine and reports the first pharmacokinetic data for the S-ODNs. This method should be applicable, with appropriate modifications, to all S-ODNs of similar molecular mass regardless of sequence.

EXPERIMENTAL

Chemicals and reagents

S-dC_{28} and S-ODN-4 were obtained from the Pharmaceutical Resources Branch of the National Cancer Institute in the form of hydrated sodium salts. The oligomers were dissolved in HPLC-grade water to provide working stock solutions of approximately 1 m*M* (2 mg in 200 μl of water). Reagents for phenol extraction were obtained from Applied Biosystems (Foster City, CA, U.S.A.; manual DNA extraction kit, Catalogue No. 401015) and these included a lysis buffer, proteinase K, a phenol reagent (phenol–water–chloroform, 70:20:10, v/v) and chloroform. The tetrabutylammonium dihydrogenphosphate (TBAP; 1.0 *M* solution in water) was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals and solvents were of analytical-reagent grade.

Equipment

A Spectra-Physics Model 8700 ternary solvent delivery system (San Jose, CA, U.S.A.) was used with a Kratos 757 variable-wavelength detector (Ramsey, NJ, U.S.A.) routinely set at 266 or 272 nm. Data were recorded and integrated for peak height and area on a Spectra-Physics Model 4200 computing integrator. Samples were introduced with either a Rheodyne Model 7125 injection valve (Berkeley, CA, U.S.A.) or a Bio-Rad Model AS-48 automatic sampler (Richmond, CA, U.S.A.) and sample volumes were typically 20 or 100 μl. A Hamilton (Reno, NV, U.S.A.) PRP-1 polymeric (styrene–divinylbenzene copolymer, 10-μm particle packing; 150 mm × 4.1 mm I.D.) reversed-phase column was used for all analytical work. A Dupont (Wilmington, DE, U.S.A.) Zorbax octadecylsilane (ODS) cartridge column (5-μm particle packing; 80 mm × 4.6 mm I.D.) was used for preliminary and comparative studies.

Chromatographic conditions

Binary gradient elution at a flow-rate of 1.0 ml/min was used. The mobile phase consisted of a mixture of acetonitrile (22.5–30%) and 0.1 *M* pH 7.0 potassi-

um phosphate buffer with 2 m*M* TBAP. Elution was accomplished with an initial 5-min isocratic period at 22.5% acetonitrile followed by a 5-min linear gradient to 30% acetonitrile, elution was continued at 30% acetonitrile for 5 min and the run was completed by returning to the initial conditions (22.5% acetonitrile) and re-equilibrating for 5 min. All analyses were done at ambient temperature with helium-sparged mobile phases.

Sample preparation

To remove interfering substances, samples were prepared by a conventional phenol extraction procedure [7] as adapted in the Applied Biosystems extraction kit. Samples (1 ml) were initially treated with the lysis buffer (1 ml) and Proteinase K (100 μl of a 25 mg/ml solution) for 30 min at 60°C. The mixture was cooled to room temperature and extracted twice with 2 ml of the phenol–chloroform–water reagent. The remaining aqueous layer was extracted twice with chloroform (2 ml) to remove excess phenol and the residual aqueous solution layer was suitable for HPLC analysis without further treatment. If larger volumes of extracted material (*e.g.* 100 μl instead of 20 μl) were injected into the HPLC system, four chloroform extractions were done. This method was suitable for analysis of either plasma or urine samples.

Protein binding estimation

Protein binding was estimated using centrifugal micropartition assemblies (Amicon Centricon-30 with a nominal relative molecular mass cut-off of 30 000 Da; W.R. Grace, Danvers, MA, U.S.A.). These ultrafiltration units retain large molecules and complexes in an upper chamber and pass smaller molecules through a separating filter to a lower chamber. Samples (1 ml) with added S-ODNs (usually at 10 μM) were centrifuged at 4°C at 5000 *g* for 30 min and the ultrafiltrate was analyzed by ultraviolet spectrometry for the presence of the oligomers. The studies with bovine serum albumim (BSA) were carried out by adding the protein to solutions of the oligomers in 0.05 *M* phosphate buffer (pH 7.0).

Exploratory pharmacokinetic study

S-dC_{28} dissolved in water for injection, was administered at a dose of 50 mg/kg (0.1-ml volume over 5–10 s) by tail vein injection to male CD_2F_1 mice (approximately 21 g; National Institutes of Health, Bethesda, MD, U.S.A.) Blood samples were obtained in heparinized tubes from the retro-orbital sinus and plasma samples processed by phenol extraction and analyzed by HPLC. Urine samples were obtained at the time of blood sampling and also after mice were housed in a metabolic cage for 24 h. Pharmacokinetic estimates were made using the AUTOMOD program of Gomeni and Gomeni [8].

RESULTS AND DISCUSSION

Ultraviolet spectroscopy confirmed the anticipated high molar absorptivities for S-dC_{28} and S-ODN-4. The maximal molar absorptivity for S-dC_{28} was 280 000 (272 nm in 0.05 *M* phosphate buffer at pH 7.0) while that for S-ODN-4 was 220 000 (266 nm). These results are consistent with estimates made by adding the molar absorptivities of the nucleotide subunits of the two oligomers. For S-dC_{28}, the molar absorptivity of deoxycytidine 5′-monophosphate is 9300 and the estimated absorptivity is 260 400. For S-ODN-4, an estimate of 238 000 is obtained by assigning the purine units an average absorptivity of 14 000 and pyrimidine units an average absorptivity of 7000 [9]. The high molar absorptivity of these oligomers clearly is an analytical advantage.

In centrifugal ultrafiltration experiments it was found that both S-dC_{28} and S-ODN-4 bind to proteins very strongly. In the absence of protein, both S-dC_{28} (M_r = 8536) and S-ODN-4 (M_r = 8909) passed readily into the ultrafiltrate. In the presence of a concentration (4%) of BSA comparable to the protein concentration present in human plasma, very small amounts of S-dC_{28} appeared in the ultrafiltrate. Taking into account non-specific binding to the filter membrane matrix, we estimate that approximately 97–98% of oligomer is protein-bound. S-ODN-4 bound extensively to the proteins present in mouse, human, rat and dog plasma as judged by retention of this oligomer on the filter membrane when plasma samples were subjected to centrifugal ultrafiltration. Binding of the S-ODN-4 (at 10 μM) to BSA was 99–100% at BSA concentrations of 0.4 and 4%; this binding is saturable since high oligomer concentrations (100 μM) resulted in appearance of relatively high concentrations (10–15 μM) of the oligomer in the ultrafiltrate.

We initially hoped to use centrifugal ultrafiltration as a sample preparation procedure, in which the oligomeric analyte would be separated from the majority of plasma proteins on the basis of membrane size selectivity. Since it was not possible to employ this approach to analyze untreated plasma by HPLC, phenol extraction was used to free the S-ODNs from the plasma proteins and remove those proteins and other interfering substances prior to analysis. The phenol extraction very effectively removes interfering substances (Fig. 1A) and allows for the ready quantitation of either S-dC_{28} (Fig. 1B) or S-ODN-4 (Fig. 1C) in mouse plasma. The large peak present in the chromatograms at a retention time of 9 min is residual phenol while the S-ODNs have retention times of approximately 12 min. By extending of the length of the linear portion of the gradient it was possible to completely resolve the S-ODNs from the phenol peak. For routine pharmacokinetic purposes it seems appropriate to retain the steeper gradient and thus keep the total analysis time at a relatively short 20 min. The recovery of oligomer in representative experiments is approximately 80% but we have noted some variability in this recovery from day to day (70–100%). Treatment of plasma samples with ethanol or acetonitrile resulted in precipitation of the oligomers

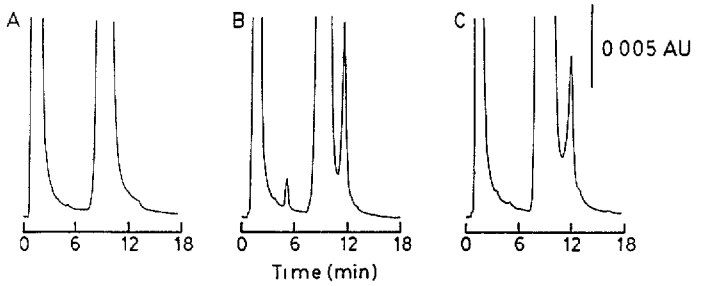

Fig 1 HPLC analysis of S-ODNs following phenol extraction from mouse plasma S-ODNs were added to mouse plasma at 37°C to a concentration of 2 0 μM, phenol-extracted and analyzed by HPLC as detailed in the text The injection volume was 100 μl and the detector was set at 266 nm (A) No S-ODN added, (B) S-dC_{28} added; (C) S-ODN-4 added.

along with proteins and other materials present in the plasma samples. We found that extraction of such precipitates with phosphate buffer (pH 7.0) led to recovery of much of the oligomer in a solution that appeared suitable for injection into the HPLC system. Unfortunately, we found that repetitive injection of these extracts into the HPLC system resulted in rapid and irreversible deterioration in column performance and we abandoned this approach to sample preparation.

The chromatographic procedure used for the S-ODNs is a modification of that reported by Makino *et al.* [10] who made the important observation that TBAP was a preferable ion-pairing agent for HPLC separation of ODNs compared to compounds with smaller alkyl groups. We also found TBAP to be an excellent ion-pairing agent. However, we were not able to satisfactorily resolve S-dC_{28} from S-ODN-4. This is consistent with the assertion of Makino *et al.* [10] that oligomer elution with TBAP is primarily dependent on base number. We also compared the performance of the polymeric column with an ODS column (Zorbax). The polymeric column gave much sharper peaks for analyses with identical mobile phases and gradient profiles, and similar column efficiencies and S-ODN capacity factors. This may reflect the influence of residual silanol groups present on the ODS column and absent on the polymeric column although other factors could be responsible.

As indicated above, it was possible to shift the retention times of the S-ODNs by variation in the gradient slope. It was not, however, possible to develop a useful isocratic separation by varying the acetonitrile concentration. In most cases either the S-ODNs did not elute or else were entirely unretained. In the best isocratic cases, the oligomers eluted immediately after the solvent front as poorly defined and quantified peaks. This indicates that the S-ODNs are trapped on the column and do not elute until a sharply defined "critical" acetonitrile concentration (approximately 28% in this case) is achieved.

This "trapping and abrupt release" phenomenon permitted the sample injection volume to be increased from 20 to 100 μl without loss of chromatographic resolution due to overloading. Use of a 100-μl sample in turn allowed the ready

measurement of samples with S-ODNs concentrations in the 20 n*M* range. Fig. 2 shows a chromatogram of 63 n*M* (100-μl injection) of S-ODN-4 for which the signal-to-noise ratio of the oligomer peak is approximately 5. Substantially greater concentration sensitivity could be achieved by further increases in the injection volume.

The expected linear relationship between oligomer concentration and peak height/area was verified for both S-dC_{28} and S-ODN-4 in water, dilute phosphate buffer or in solutions obtained following phenol extraction. The correlation coefficients in all cases was greater than 0.99 up to 0.2 nmol injected (2 μM). The reproducibility of the analysis method is very good in the course of a day for both extracted and non-extracted samples In a test, eight repeat injections of S-ODN-4 sample (10 μM in water; 20-μl injection) gave a retention time of 12.06 ± 0.04 min and a coefficient of variation in peak-height quantitation of 3.3%. A similar test of repetitive analysis of four samples of both S-dC_{28} and S-ODN-4 (1μM in mouse plasma, 100-μl injection) gave very good results. The retention times for the two oligomers were 11.64 ± 0.07 and 12.43 ± 0.01 min, respectively, and coefficients of variation in peak height and extraction efficiencies were 3 and 1%, respectively. We have not been able to identify an appropriate internal standard for the analysis of these oligomers, and external standard solutions of the oligomers (processed as appropriate for the samples to be analyzed) must be used for analytical calibration.

Both S-dC_{28} and S-ODN-4 were relatively stable in solution. Following incubation in either plasma or dilute pH 7.0 phosphate buffer for 24 h at 37°C, we observed a loss of less than 10% of S-ODN-4 (in the 2–10 μM range); S-dC_{28} showed approximately 10% loss when incubated under comparable conditions in mouse plasma but little, if any, loss was observed in phosphate buffer.

A preliminary pharmacokinetic study was performed with S-dC_{28} and the results are reported in Table I. The concentration of the oligomer in plasma 10

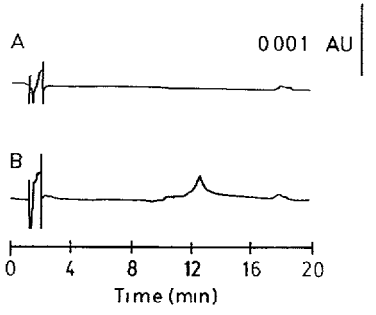

Fig 2 HPLC analysis of a 63 n*M* aqueous sample of S-dC_{28} HPLC analysis was performed as detailed in the text with an injection volume of 100 μl and detection at 266 nm (A) Water blank, (B) 63 n*M* S-dC_{28} in water The oligomer peak has a retention time of approximately 13 min and a signal-to-noise ratio of approximately 5

TABLE I

ESTIMATED PHARMACOKINETIC PARAMETERS FOR S-dC_{28} FOLLOWING INTRAVENOUS ADMINISTRATION TO MICE (50 mg/kg)

Parameter	Value
Elimination half-time (min)	11.6
Area under the curve (μmol min/l)	556 0
Volume of distribution (ml)	3 0
Total body clearance (ml/min)	0 2

min after drug administration was 17 μM. The rapid elimination of the drug (elimination half-time of 11.6 min) was initially surprising considering the extensive protein binding of the oligomer as well as its relatively large size. Observation of high (μM) concentrations of S-dC_{28} in urine samples taken within the first hour following drug administration indicate rapid renal excretion, probably due simply to glomerular filtration. Apparently the "dynamic" situation *in vivo*, with glomerular filtration constantly removing the small amount of free oligomer, minimizes the pharmacological importance of the extensive protein binding observed in the "static" situation *in vitro*. It should be noted that the efficient clearance of negatively charged dextran derivatives with molecular masses in the 10 000–20 000 Da range appears to provide a parallel to our observations [11].

Although our study is preliminary, it is of significance that we estimate that approximately 75% of the oligomer will be cleared by renal excretion within the first 4 h. Since S-ODNs are extremely expensive to synthesize and are currently available in very limited quantities, this data should be of value in making optimal use of S-ODNs in further pharmacokinetic and other studies.

CONCLUSIONS

We have demonstrated that ion-pair reversed-phase HPLC using ultraviolet detection is sufficiently sensitive to detect S-ODNs at concentrations as low as 20 nM in a total analysis time of 20 min. The use of a large sample volume contributes substantially to the enhanced sensitivity of our assay while the chromatographic properties of the S-ODNs avoid the problems of sample overloading typically seen with such large sample volumes. We have demonstrated that phenol extraction represents a good approach to preparation of plasma samples. The method removes virtually all interfering substances, results in good recovery of S-ODNs (70–100%) and is characterized by excellent within-day reproducibility. We have found that S-ODNs are relatively stable in plasma and have also found that at least one oligomer (S-dC_{28}) is rapidly cleared from blood, probably by glomerular filtration. This property is likely to be shared by all S-ODNs of similar molecular mass and has implications for future use of these compounds in chemotherapy.

ACKNOWLEDGEMENTS

This work was supported by National Cancer Institute contract No. N01-CM-87286 and by the National Cancer Institute Vermont Regional Cancer Center Core Grant PHS CA-22435-09.

REFERENCES

1 P S. Miller and P O. P Ts'o, *Ann Reports Med Chem*, 23 (1988) 295
2 G Zon, *Pharm Res*, 5 (1988) 539
3 E De Clercq, F Eckstein, H Sternbach and T C. Merigan, *Virology*, 42 (1970) 421.
4 M Matsukura, K Shinozuka, G Zon. H Mitsuya, M Reitz, J S Cohen and S Broder, *Proc. Natl. Acad Sci U S A*, 84 (1987) 7706.
5 S. Agrawal, J. Goodchild, M. P Civeira, A. H Thornton, P. S. Sarin and P C Zamecnik, *Proc Natl. Acad. Sci U S A.*, 85 (1988) 7079
6 C A. Stein, C Subasinghe, K. Shinozuka and J S Cohen, *Nucleic Acids Res*, 16 (1988) 3209
7 L G. Davis, M D Dibner and J. F Battey, *Basic Methods in Molecular Biology*, Elsevier, Amsterdam, New York, 1986, pp. 44–46
8 R Gomeni and C. Gomeni, *Comput Biol Med*, 9 (1979) 39
9 C J Marcus-Sekura, *Anal. Biochem.*, 172 (1988) 289
10 K Makino, H Ozaki, T. Matsumoto, H Imaishi, T. Takeuchi and T Fukui, *J Chromatogr*, 400 (1987) 271
11 E Koushanpour and W Kris, *Renal Physiology*, Springer-Verlag, New York, 1986, p 65.