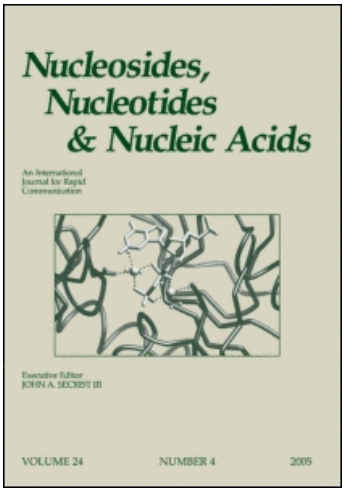

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ANTI-BCL2 PHOSPHOROTHIOATE G3139 CLINICAL PHARMACOLOGY: PLASMA LEVELS DURING CONTINUOUS SUBCUTANEOUS INFUSION BY HPLC ASSAY

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ABSTRACT: An HPLC assay has been developed to determine plasma levels of G3139 - a 18mer phosphorothioate oligonucleotide currently in Phase I clinical studies. The assay utilizes anion exchange microchromatography, aqueous LiBr gradient elution, and UV absorbance detection. Minimum sensitivity of approximately 0.2 μg/ml plasma, or 35 nM of G3139, has been achieved. Analysis of preliminary clinical samples indicates subcutaneous infusion of G3139 at 2 mg/kg/day gives rise to steady state plasma levels of 1-2 μg/ml.

INTRODUCTION

An 18-mer phosphorothioate oligodeoxynucleotide analogue, G3139, has been shown to down regulate the apoptosis inhibiting protein BCL2 and inhibit human tumor xenograft animal models[1]. This 18-mer has been evaluated in pharmacology and toxicology studies using continuous subcutaneous infusion for 14 days of a saline solution to define the MTD and plasma pharmacokinetics[2]. Here we describe an improved HPLC assay[3] for G3139 detection in plasma samples achieving minimum sensitivity of approximately 0.2 μg/ml and requiring as little as 25 μL of plasma sample.

METHODS AND MATERIALS

Chemicals. "Cold" G3139 and "hot" [^{35}S]-G3139 phosphorothioate oligomers were synthesized, isolated and purified by standard procedures. High purity Tris base and LiBr were used. Water and HBr were distilled. The buffer solutions for the HPLC assay were as follows: 25 mM Tris-HBr, pH 7.0 (buffer A); 2M LiBr in 25 mM Tris-HBr, pH 7.0 (buffer B).

HPLC Analysis. The analyses were performed on a Beckman Gold HPLC system equipped with a model 166 UV detector. Radioactivity was detected by a Packard RadioMatic Flow Scintillation Analyzer installed after the UV detector. The microcolumn was a C-130-B 2 x 20 mm Precolumn (Upchurch Scientific, Inc., Oak Harbor, WA) packed with Pharmacia 15Q anion-exchange resin. The gradient was composed of buffer B in buffer A as follows: 0.0 to 0.5 min. - 12.5% fixed ; 0.5 to 7.34 min. - from 12.5 to 87.5% ; 7.34 to 8.34 min. - from 87.5 to 12.5% ; 8.34 to 18.0 - 12.5% fixed.

Preparation of G3139 Plasma Samples for Calibration and Analysis.

For calibration typically an aliquot of G3139 (or [^{35} S]-G3139) solution with known concentration was mixed with 100 μL of the defrosted plasma, 25 μL of 8X buffer (0.2 M Tris-HBr, 2.0 M LiBr, pH 7.0) and diluted with distilled water up to 200 μL of total volume. The mixture was centrifuged for 5 min. at 14000. Method development evaluated injection volumes ranging from 5 μL to 80 μL. For routine analysis of G3139, level typically an aliquot of 100 μL of human or monkey plasma was mixed with 25 μL of 8X buffer and 75 μL of distilled water and analyzed as above with an injection volume of 50 μl.

RESULTS

The LiBr gradient results in an increasing baseline with UV absorbance detection (Fig. 1A,B). Without exceptions, the plasma protein components elute near G3139 which has a retention time of 5 min. but either earlier or later than it. These background problems are overcome by simple subtraction of a blank run (for example: sample of plasma from the same individual prior to G3139 administration) (Fig. 1C). The absence of significant binding of G3139 to plasma proteins under the assay conditions has been confirmed by radioactivity detection (Fig.1D). Note that a separation of full length G3139 and (N-2) oligomer does not occur under these conditions chosen for routine analysis (7 min. gradient), but can be achieved by expanding the gradient to 20 min, with some loss in sensitivity. Standard calibration curves (Fig. 2) were generated by serial dilution of G3139 (and/or [^{35} S]-G3139) in human plasma. Both the amount of oligomer injected (1 - 320 ng) and the injection volume (5 - 80 μL) were varied. Fig. 1A shows the dependence of the absorbance at the maximum of the G3139 peak *vs* its concentration. Fig. 1B shows the dependence of radioactivity *vs* the G3139 concentration. The linearity of both dependencies and the correlation between them (Fig. 2C) are satisfactory in the range of 10 ng to 320 ng of G3139. Present sensitivity of the assay was estimated to be 5 ng of G3139 in 50 μL injection or 0.2 μg/ml plasma.

Table shows the level of G3139 in limited human and monkey plasma samples available from ongoing clinical studies. It is seen that continuous subcutaneous

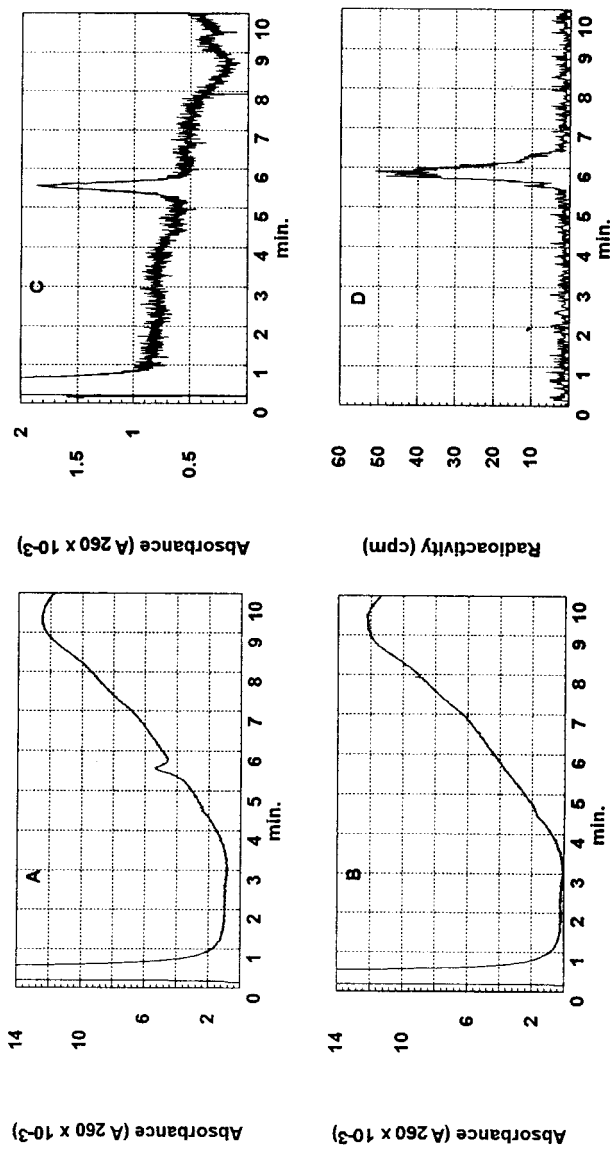

Figure. 1 HPLC of [^{35}S]-G3139 spiked in human plasma. Injected 10 ng of oligomer.
A - trace of absorbance of sample containing [^{35}S]-G3139. B - trace of absorbance of blank sample (without [^{35}S]-G3139). C - subtraction trace: trace (A) - trace (B).
D - trace of radioactivity of sample containing [^{35}S]-G3139.

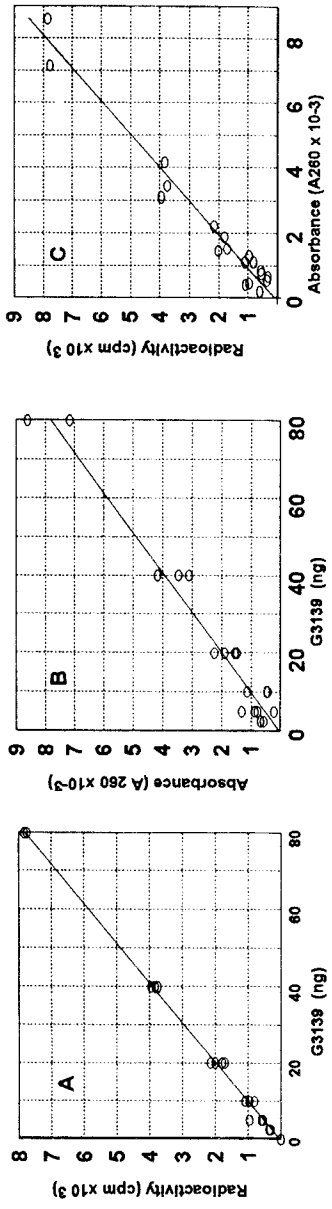

Figure 2. Standard calibration curves for G3139 HPLC assay.
A - radioactivity in the peak of [^{35}S]-G3139 vs contents of oligomer in plasma sample. B - absorbance at the maximum of the peak of [^{35}S]-G3139 vs contents of oligomer in plasma sample. C - correlation between radioactivity and absorbance.

TABLE: G3139 levels in human and monkey plasma by HPLC assay

Continuous subcutaneous infusion*		Dose level, mg/kg/day	Day of the sample acquisition	G3139 concentration, microgram/mL
Human:	Patient-8	2.0	2	2.7 (± 0.5)**
			7	1.6 (± 0.3)
Monkey:	Animal-2	1.0	3	0.41 (± 0.1)
			9	0.50 (± 0.1)
	Animal-3	10.0	3	3.1 (± 0.3)
			7	15.1 (± 1.5)
			9	25.3 (± 2.5)

* Details will be published elsewhere. ** Estimated error of G3139 level determination.

administration of G3139 at 1-10 mg/kg/day to monkeys leads to 0.5-25 μg/mL of oligomer in plasma. These values are characteristic of the therapeutic levels of G3139 in murine tumor models. Surprisingly, we did not detect shorter products of G3139 degradation in patient plasma. Nonetheless, we have shown that shorter oligomers can be easily detected by hydrolysis of G3139 with S1 nuclease.

CONCLUSIONS

An HPLC assay has been developed to determine levels of G3139 in patient plasma with a routine sensitivity down to 0.2 μg/ml (or 35 nM) requiring as little as 25 μL of plasma for analysis. It is based on microcolumn anion exchange chromatography and UV absorbance detection allowing direct injection of plasma (serum) sample without any pretreatment of the sample other than dilution and centrifugation. Any binding to plasma proteins appears to be almost completely eliminated in the chromatography conditions used and does not exceed 5-7% of total G3139 contents in the sample. The method developed appears reproducible, reliable, and cost effective.

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