ELSEVIER

Journal of Chromatography B, 750 (2001) 129–135

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Detection of a mixed-backbone oligonucleotide (GEM 231) in liver and tumor tissues by capillary electrophoresis

Reeta Bansal[a], Helen X. Chen[b], John L. Marshall[b], Jie Tan[b], Robert I. Glazer[b], Irving W. Wainer[a,*]

[a]*Bioanalytical Center, Department of Pharmacology, Georgetown University Medical Center, 3900 Reservoir Road NW, Washington, DC 20007, USA*

[b]*Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007, USA*

Received 26 April 2000; received in revised form 28 July 2000; accepted 28 July 2000

Abstract

A simple, rapid and sensitive method has been developed and validated for the analysis of a mixed-backbone oligonucleotide (GEM 231) in tumor tissues. The analysis was performed using a capillary electrophoresis (CE) system with UV detection. An extended light path (bubble cell) capillary column of 64.5 cm (effective length 56 cm)×50 μm I.D. is used as the separation column. The optimized chromatographic conditions were background electrolyte: sodium borate buffer (60 m*M*, pH 9.1), electrokinetic injection: 10 s, applied voltage: 30 kV, detection at λ=210 nm. A linear relationship was observed between the peak area and the amount of GEM 231 in the range of 1.0–1000 μg/ml. The lower detection limit of the drug was 100 pg with an average recovery of about 75±5%. The inter-day and intra-day relative standard deviations were <10%. Assay validation studies revealed that CE method is reproducible and specific for the determination of GEM 231 in tissue homogenates with a run time of less than 5 min.

Keywords: GEM 231; Oligonucleotides

1. Introduction

In recent years antisense oligonucleotides have emerged as promising therapeutic agents for treatment of viral infections and cancer [1,2]. The ability to hybridize with target mRNA from an essential gene offers the means to modulate cell replication that can be used for the therapeutic suppression of oncogene expression and viral replication.

Antisense oligonucleotides must be modified to prevent enzymatic digestion [3–8]. The most extensively studied modifications involve oligodeoxynucleotides phosphorothioate (PS-oligos), in which one of the non-bridged oxygen atoms of the internucleotide linkage is replaced with a sulfur atom [5,6,8,9]. Although PS-oligos have demonstrated promising therapeutic potential and better stability, their use has been restricted by their dose dependent toxicity and low binding affinity to the target mRNA. A common side effect of PS-oligos is their stimulatory effect, which is attributed to their polyanionic nature [9–11].

In order to avoid the problems associated with the PS-oligos, second generation oligonucleotides called

*Corresponding author. Tel.: +1-202-6871-650; fax: +1-202-6875-015.

E-mail address: wainer@gunet.georgetown.edu (I.W. Wainer).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00436-9

mixed backbone oligonucleotides (MBOs) have been designed [5,9]. MBOs contain segments of PS-oligos and appropriately placed segments of modified oligodeoxynucleotides or oligoribonucleotides, and have advantage of both the PS-oligo and the modified oligodeoxynucleotide. In MBOs, the choice of oligonucleotide modification and its placement are critically important. In animal models, some of the MBOs have indicated improved properties compared to PS-oligos, such as, better affinity to RNA, improved in vivo stability and oral bioavailability, and markedly improved dose-related side effects [12].

GEM 231 (Fig. 1) [29] is a prototypic mixed-backbone oligonucleotide targeting the regulatory subunit α of type I protein kinase A (PKA-RIα). It is an 18-mer antisense oligonucleotide containing a PS-oligo backbone with terminal 2′-*O*-methylribonucleoside modification. GEM 231 is currently being clinically evaluated in one of the first studies of mixed backbone oligonucleotide in cancer patients [13,14]. In connection with this study, we have been developed an assay to measure MBO in biological samples.

Capillary gel electrophoresis (CGE) is emerging as a preferred method in quantifying intact oligonucleotides as well as their putative metabolites [15–17]. However, the existing gel columns are not very stable under biological media making a CGE assay very time-consuming [17–21]. The prevalent approach is to use high-performance liquid chromatography (HPLC) to quantify the total amount of antisense compounds, and CGE to determine the relative proportion of the intact and metabolic species [21,22]. Free solution capillary electrophoresis (CE) is another powerful technique that could be effectively employed to quantify the total amount of antisense compounds from biological samples. Lower mass sensitivity and short analysis time make it preferable to HPLC, especially if the quantities of available tissue samples are small and concentrations of the drug are low.

We have developed a CE assay for the quantitation of GEM 231 in tumor tissues from nude mice. There are very few existing methods for the measurement of low levels of oligonucleotides in biological fluids [23–26], and no method has been previously re-

Drug Structure

Where, the base sequence is: GCGUGCCTCCTCACUGGC

A= 2' – deoxyriboadenosine	A= riboadenosin
C = 2' – deoxyribocytidine	C= ribocytidine
G = 2' – deoxyriboguanosine	G = riboguanosine
T = thymidine	U = uridine

The underlined bases are 2' – methoxy ribose substituted oligonucleotides

Fig. 1. Drug structure. GEM 231 is the sodium salt of 3′→5′ linked 18-mer RNA/DNA hybrid phosphorothioate oligonucleotide in which each of the 17 internucleotide linkages is a mixture of diastereomers. Base sequence: 5′-GCGUGCCTCCTCACUGGC-3′ is complementary to the mRNA of N-terminal codon 8-1 of the RIα subunit of PKA-I. The underlined bases on the 5′- and 3′-ends have ribose sugars modified with a 2′-*O*-methyl group; the 17 internucleotide linkages are *O*-linked phosphorothioate. GEM 231 has a molecular mass of 6287 u in its 17 Na+ salt form and 5913 as the free acid.

ported for the measurement of GEM 231 in tissue homogenates. The assay reported here is simple, rapid, sensitive, and can be used in in vivo studies.

2. Experimental

2.1. Materials

All reagents used were of analytical grade, and were used without further purification. Sodium tetraborate, was obtained from Aldrich (Milwaukee, WI, USA). Phenol, chloroform, acetone, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Nonidet P-40, proteinase K and other necessary reagents were obtained from Sigma (St. Louis, MO, USA). The compound GEM 231 (HYB0165) and internal standard (poly-deoxynucleotide-T27-mer phosphorothioate) were obtained in the lyophilized form from Hybridon (Milford, MA, USA), and the drugs were stored at −28°C, until use.

The capillary column was an analytical extended light path capillary purchased from Hewlett-Packard (HP) (Wilmington, DE, USA). Solid-phase strong anion-exchange cartridges (Accubend, SAX-SPE 100 mg) were procured from J&W Scientific (Pleasenton, CA, USA) and solid-phase C_{18} endcapped cartridges (Isolute, RP-SPE 100 mg) were from International Sorbent Technology (Lakewood, CO, USA).

2.2. Stock solutions

Stock solutions of GEM 231 and internal standard, poly-deoxynucleotide-T27-mer 1.0 mg/ml were prepared by dissolving appropriate amounts in normal saline. All stock solutions could be stored at 4°C for 4 weeks without any degradation. The stock solutions were diluted for preparation of assay standards.

2.3. Background electrolytes

A variety of background electrolytes were examined including citrate, acetate, phosphate, Tris and sodium borate buffers. The optimum pH was determined using a fixed 60 m*M* sodium borate concentration and by varying the pH from 4.0 to 10.0. As the pH increased, the running time decreased. Based on the results, the pH of the running buffer was set at 9.1, where the run time was less than 5.0 min and the peaks of interest were resolved from matrix interferences.

2.4. Separation buffer

The electrolytic buffer solution was sodium borate (60 m*M*, pH≈9.1) prepared in deionized water (Millipore – Milli-Q ultrapure water system; Bedford, MA, USA). The resistance of the water was more than 18.0 $M\Omega/cm^3$. The buffer solution was filtered through a 0.45-μm, filter (Millipore) degassed and sonicated under vacuum prior to use.

2.5. Tissue collection

In order to test the feasibility of measuring GEM 231 within the tumor tissues, a preliminary experiment was performed in nude mice (Taconic, NY, USA), aged 4–5 weeks and weighing 20–30 g, using a protocol that simulated the clinical trial. Human colon cancer cell line HCT-15 was implanted subcutaneous in nude mice. Upon growth of the tumor to sizes of about 500 mg, GEM 231 was administered by intravenous (IV) bolus at doses of 10 mg/kg, 20 mg/kg and 50 mg/kg. Mice were sacrificed 48 h following GEM 231 injection. During the surgical resection, a portion of both resected normal liver and tumor tissues (50–100 mg each) was collected and frozen immediately in liquid nitrogen for the measurement of drug levels. The frozen samples were stored at −80°C until analysis.

2.6. Instrumentation

All capillary electropherograms were obtained with a Hewlett-Packard 3D capillary electrophoresis system with diode-array detector and HP 1100 pumps (Waldbronn, Germany). An extended light path (bubble cell) capillary of 64.5 cm (effective length 56 cm)×50 μm I.D. was used as a separation column. The capillary was mounted in a cartridge, which was thermostated at a temperature of 25°C. Samples were introduced by an electrokinetic injection at 30 kV for 10 s. Detection measurements were performed by UV at λ=210 nm and the data were collected using the HP software ChemSation.

2.7. Capillary preparation

New capillaries were conditioned by performing the following flushes: pressure injection of 0.1 *M* NaOH for 5 min, water for 5 min, and then equilibration with running buffer for 5 min. Prior to each run, and between the runs, the capillary was purged with 0.1 *M* NaOH for 2 min and then with running electrolyte (60 m*M*, pH≈9.11) for 5 min.

2.8. Preparation of tissue samples for CE analysis

Samples were prepared using a modification in the protocol developed by Phillips et al. [27]. Tissue samples 50 mg each of liver or tumor were used for extraction. Samples were homogenized in Tris–HCl buffer (20 m*M*, pH 8.0), 20 m*M* EDTA, 0.1 *M* NaCl, 0.5% nonionized detergent P-40, containing 1.0 μg/ml I.S. Since liquid–liquid extraction (phenol/chloroform) did not improve peak resolution or recovery, this step was not carried out and the homogenate was treated sequentially with proteinase K and 30% NH_4OH. The resulting solution was evaporated to dryness at 55°C. The dried samples were resuspended in 5 ml of the loading buffer [Tris–HCl (10 m*M*, pH 9.0), 0.5 *M* KCl, 20% (v/v) acetonitrile] and passed through a SAX-SAP. The column was washed with 3.0 ml of loading buffer and eluted with 3.0 ml of elution buffers composed of Tris–HCl (10 m*M*, pH 9.0) containing 0.5 *M* KCl, 1.0 *M* NaBr, and modified with acetonitrile (30%, v/v). The SAX-SPE elute was loaded onto the RP-SPE and washed with 5 ml of deionized water, and eluted with 4.0 ml of 20% (v/v) aqueous acetonitrile. The RP-SPE elute (sample) was concentrated under vacuum, and reconstituted with 100 μl electrophoretic buffer. No further desalting was considered necessary, as there was no coelution from matrix. Samples were injected for 10 s with a voltage of 30 kV.

3. Results and discussion

The analytical method has been developed and validated for the measurement of second-generation (GEM 231) xenograft tissue samples. The electrophoretic separations of GEM 231 were carried out with an electrolyte, sodium borate buffer, 60 m*M* at pH≈9.11. Each injection volume was calculated to be 99.4 nl by using the Paiseuille equation [28]. Using the described conditions, the analysis was completed in less than 5 min with a migration time of 3.0±0.3 min for GEM 231 and for 3.5±0.3 min for T27 (Fig. 2A).

The calibration curve was found to be linear over the concentration range 1.0–1000.0 μg/ml. The linear regression equation ($y=mx+c$) obtained was: $y=1525.7x$, where, y is the peak area in mAU and x is the concentration in μg/ml and c is intercept. The coefficient of determination (r^2) was 0.999. The Student's *t*-test conducted for testing the strength of relationship between the peak area and the concentration showed a P value <0.0001.

The precision of the analysis was evaluated within-day ($n=3$) and between-day ($n=3$) in liver tissue samples. The assay validation data are summarized in Table 1. The variability was expressed as the percentage relative standard deviation (RSD) and ranged from 6.6 to 8.7% in liver homogenates. The limit of quantitation was 100 pg with a recovery of 75±5%. The tissue sample preparation method was convenient and reproducible. There were no endogenous interferences with the drug in the samples (Fig. 2B) and no evidence of GEM 231 degradation during sample preparation. It was also found that drug was stable in electrolyte buffer at room temperature during the day.

3.1. Detection of GEM 231 in cancer xenograft following a single i.v. injection in mice

Xenograft tumor tissues, 50–100 mg were homogenized and the concentration of GEM 231 was measured by CE (Fig. 2C). At a dose of 20 mg/kg, the tissue concentrations of GEM 231 at 48 h were 14.5 μg/g in tumor and 22.7 μg/g in the liver and at the dose of 50 mg/kg the drug concentration in liver was found to be 63.0 μg/g (wet tissue weight). This result is similar to that previously reported for 35-S-GEM 231 [27]. Notably, unlike the first-generation antisense oligonucleotides (AON) where only 40% of the oligonucleotides was intact at 24 h, all of the GEM 231 molecules remained in the unmodified form at 48 h post injection.

Based on our study in the animal model, a tissue concentration of 14.5 μg/g in tumor xenograft can

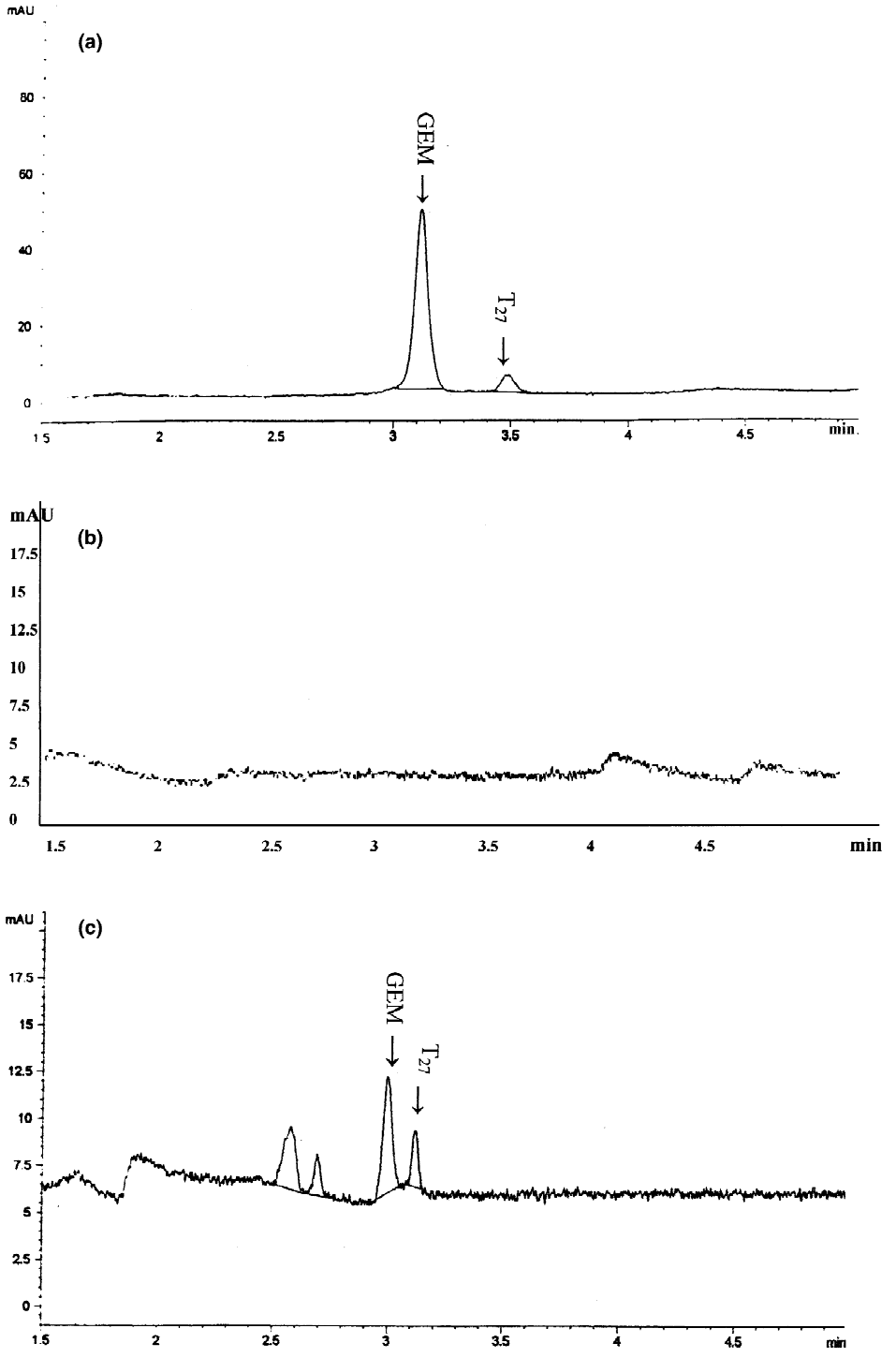

Fig. 2. (A) Electropherogram of GEM 231 (50 μg/ml) and I.S. (1 μg/ml) dissolved in normal saline solution. (B) Blank extracted tissues. (C) Extracted xenograft tumor tissues with the concentration of 14.5 μg/ml at 48 h after i.v. bolus injection of 20 mg/kg GEM 231.

Table 1
Validation of CE assay of antisense oligonucleotide (GEM 231) in tissue homogenates of nude mice[a]

Sample No.	Added concentration (μg/ml)	Inter-day		Intra-day	
		SD (μg/ml)	RSD (%)	SD (μg/ml)	RSD (%)
1	100	±8.03	8.73	±3.08	3.09
2	50	±3.19	6.25	±0.33	0.66
3	10	±0.96	8.67	±0.47	4.80

[a] $n=3$, SD=standard deviation, RSD=relative standard deviation.

be achieved following a single injection of 20 mg/kg (60 mg/m^2). Since the lower detection limit was 100 pg, it is expected that by analyzing a tissue size of 50 mg, GEM 240 mg/m^2 in patients should be within the detectable range.

4. Conclusions

Extensive development of antisense drugs and the introduction of advanced second-generation oligonucleotides are an exciting new class of therapeutic agents, that have the potential to rapidly capitalize on the expanding body of human genome sequence data. However, we are still far from full realization of this therapeutic potential and there is a need for reliable analytical methods to measure the second-generation drugs. We have developed an automated CE–UV method for the analysis of mixed backbone target drug (GEM 231) in liver and tumor tissue samples that may also be applicable to other biological samples. The assay provides simple, rapid, specific and reproducible detection and measurement of GEM 231 in xenograft tissue samples.

Acknowledgements

Financial support for this work was provided by fellowship award to H.X.C. of Translational Research from American Association of Cancer Research and Clinical Research Award from Cancer Leukemia group B (CALGB) Foundation.

References

[1] P.C. Zamecnik, in: Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, Wickstrom–Wiley–Liss, New York, 1992, p. 1.
[2] S. Agrawal, Trends Biotechnol. 14 (1996) 376.
[3] S. Agarwal, Q. Zhao, Curr. Opin. Chem. Biol. 2 (1998) 519.
[4] S. Agrawal, Antisence Therapeutics, Humana Press, Clifton, NJ, 1996.
[5] S. Akhtar, S. Agarwal, Trends Pharm. Sci. 18 (1997) 12.
[6] E. Wickstrom, Clinical Trials of Genetic Therapy with Antisense DNA and DNA Vectors, Marcel Dekker, New York, 1998.
[7] C.A. Stein, A.M. Kreig, Applied Antisense Oligonucleotide Technology, Wiley–Liss, New York, 1998.
[8] C.F. Bennett, Biochem. Pharmacol. 55 (1998) 9.
[9] S. Agarwal, Q. Zhao, Z. Jiang, C. Oliver, H. Giles, J. Heath, D. Serota, Antisense Nucleic Acid Drug Dev. 7 (1997) 575.
[10] S.P. Henry, P.C. Giclas, J. Leeds, J. Pangburn, M. Auletta, C.A.A. Levin, D.J. Kornbrust, J. Pharmacol. Exp. Ther. 281 (1997) 810.
[11] D.R. Shaw, P.K. Rustagi, E.R. Kandimalla, A.M. Manning, Z. Jiang, S. Agarwal, Biochem. Pharmacol. 53 (1997) 1123.
[12] T. Giampaolo, C. Rosa, P. Grazia, P. Stefano, A.R. Bianco, S. Agarwal, J. Mendelsohn, F. Ciardiello, Clin. Cancer Res. 5 (1999) 875.
[13] H. Chen, J. Marshall, E. Ness, R. Martin, B. Dvorchik, N. Rizvi, M. Mekinlay, W. Dahut, M.J. Hawkins, Clin. Cancer Res. 6 (2000) 1259.
[14] H. Chen, J. Marshall, E. Ness, R. Martin, B. Dvorchik, N. Rizvi, M. McKinlay, W. Dahut, M.J. Hawkins, Clin. Cancer Res. (1999) in press.
[15] A.S. Cohen, A.J. Bourque, B.H. Wang, D.I. Smsek, A. Belenky, Antisense Nucleic Acid Drug Dev. 7 (1997) 13.
[16] A.S. Cohen, M. Vilenchik, J.I. Dudley, M.W. Gemborys, A.J. Bourque, J. Chromatogr. 638 (1993) 293.
[17] A.J. Bourque, A.S. Cohen, J. Chromatogr. 617 (1993) 43.
[18] A.J. Bourque, A.S. Cohen, J. Chromatogr. B 662 (1994) 343.
[19] I.D. DeDiontsto, D.H. Lloyd, J. Chromatogr. A 735 (1996) 191.
[20] M.A. Gilar, D.I. Belenky, A. Smtsek, A.J. Bourque, Nucleic Acids Res. 25 (1997) 3615.

[21] K. Makino, H. Ozaki, T. Matsumoto, H. Imaiishi, T. Takeuchi, J. Chromatogr. 400 (1987) 271.
[22] A. Pompon, I. Lefevre, J.L. Imbach, Biochem. Pharmacol. 43 (1992) 1769.
[23] K. Khan, K. Liekens, A.V. Aerschot, A.V. Schepdael, J. Hoogmartens, J. Chromatogr. B 702 (1997) 69.
[24] A. Khan, V. Schepdael, J. Hoogmartens, J. Chromatogr. A 742 (1996) 267.
[25] K. Khan, W. Talmadge, M. Zhu, I. Olech, C. Siebert, J. Chromatogr. A 744 (1996) 347.
[26] S.H. Chen, M. Qian, J.M. Bernnan, J.M. Gallo, J. Chromatogr. B 692 (1992) 43.
[27] J.A. Phillips, S.J. Craig, D. Bayley, R. Christian, A.R. Geary, P.L. Nicklin, Biochem. Pharmacol. 54 (1997) 657.
[28] J. Harbaugh, M. Collect, H.E. Schwartz, Technical Information Bulletin TIBC-103, Beckman Instruments, Fullerton, CA, 1989.
[29] S. Agarwal, R. Zhang, G. Tortora, Y.S. Cho-Chung, Hybridon GEM 231. Investigator Brochure, MA, Nov. 1998, 13.