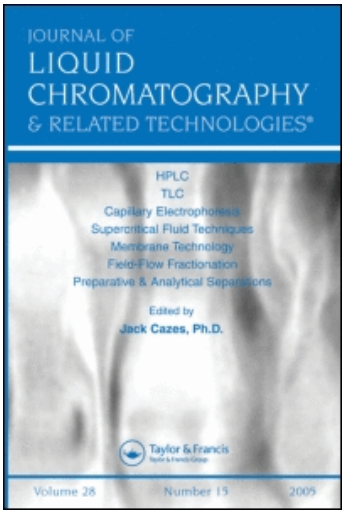

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Study of the Stability of Oligodeoxynucleotide-Doxorubicin Conjugate In Vitro and Its Pharmacokinetics In Vivo by RP-HPLC

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Study of the Stability of Oligodeoxynucleotide–Doxorubicin Conjugate In Vitro and Its Pharmacokinetics In Vivo by RP-HPLC

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ABSTRACT

Oligodeoxynucleotide–doxorubicin conjugate is a novel modified oligodeoxynucleotide (ODN) to inhibit the expression of *mdr1* gene. The present study was undertaken to determine the stability of the conjugate in vitro and its pharmacokinetics in vivo using a reverse-phase HPLC assay. The method employed a short C_{18} reverse phase column combined with a C_8 pre-column and a linear gradient elution with acetonitrile

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containing 0.1 M aqueous triethylammonium acetate, pH 7.0. Detection was carried out using a UV-diode array detector at 254 and 480 nm. Minimum sensitivity of ~0.15 μg/mL in plasma and 0.1 μg/mL in PBS of the conjugate was achieved. After incubation in 10% activated fetal calf serum for 24 hours, 15.8% of the conjugate was degraded. As a comparison however, 97.2% of the control ODN was degraded within the same incubation time. The pharmacokinetics studies show that the half-lives of the conjugate is about 8 hours, 4 times longer than ODN as a control. Assay validation studies revealed that the method is accurate, reproducible for determination of the conjugate, and can be used for a pharmacokinetic study of the conjugate.

Key Words: Oligodeoxynucleotide-doxorubicin conjugate; Pharmacokinetics; *mdr1*; Stability.

INTRODUCTION

Oligodeoxynucleotides (ODNs) are able to efficiently regulate gene expression by inhibiting transcription or translation via sequence-specific binding of either DNA or RNA, respectively.[1–3] They have been a potentially powerful tool for the therapeutic manipulation of genes associated with cancer. However, the clinical efficiency of unmodified ODN is limited because it is degraded rapidly by nucleases in living systems. To overcome this, various modifications of ODN have been developed.[4–8] One major modification is coupling ODN with intercalators such as acridine, adriamycin, psoralen, and pyrene. A novel, modified ODN that coupled ODN with doxorubicin (DOX) at 3′-end was synthesized in our laboratory. The conjugate is expected as an antisense drug to inhibit the expression of the *mdr1* gene that induces the multidrug resistance (MDR) phenomenon. It is important to develop convenient method detection for the levels of the conjugate in vitro and in vivo for future studies of the conjugate. In general, the analysis of ODN includes electrophoresis,[9] radiochemical,[10,11] hybridization,[12] chromatographic[13,14] methods. These methods have some defects, such as lack of sensitivity, inconvenience, and maybe changing properties of ODN (using radio-labeled).[15] Recently, an anion-exchange HPLC and capillary gel electrophoresis (CGE) assay has been developed for quantitative analysis of ODN in biological fluids.[16,17] Here, we describe a simple, sensitive, reliable, and reproducible RP-HPLC method, which uses two-fold detection for the direct quantitative determination of the stability of ODN–DOX conjugate in plasma in vitro and for its pharmacokinetic studies in vivo.

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EXPERIMENTAL

Instrumentation

Agelent 1100 series system equipped with a Model 1100 Quat pump system, a Model 1100 autoinjector, and a Model 1100 diode-array variable-wavelength UV detector (Agilent Technologies, Germany) was used. The chromatograms were analyzed with a chromatographic workstation (Agilent 1100). Precolumn (Agilent C_8, 5 μm, 4.6×20 mm) was purchased from Agilent Technologies (Germany). A RP column (Lichrospher 5 μm, 4.6×125 mm) was purchased from Merck (Germany). Columns were thermostated at 25°C and protected by filters which were changed daily.

Chemicals and Reagents

The sequence of ODN is 5′-TCCTCCATTGCGGTCCCCTT-3′, the 30-11 region of *mdr1* gene resulting MDR in a human epidemic carcinomata cell line, which could control expression of the gene.[8] ODN and ODN with a phosphate group at the 3′-end, were purchased from Shanghai Bioasia Biotech Co. Ltd. (Shanghai, China). DOX was a gift from Zhejiang Hisun Pharmacentical Co. Ltd. (Zhejiang, China). The conjugate of ODN–DOX was synthesized and purified in our laboratory. Fetal calf serum and heat-inactivation were purchased from Shanghai Bioasia Biotech Co. Ltd. (Shanghai, China).

Distilled water was purified on a Milli Q system (Millipore, Bedford, MA). The resistance of the water was more than 18.0 MΩ/cm^3. Triethylamine, acetic acid, and acetonitrile were all HPLC grade and were purchased from Merck (Germany).

Animals (healthy male Spraghe-Dawley rats, 100–120 g) were purchased from SIPPR/BK, (Shanghai, China) and were fed with a commercial diet and water in our laboratory for 1 week prior to the study.

Chromatographic Conditions

The mobile phase included two eluents. Eluent A: 5% CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7.0. Eluent B: 80% CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7.0. The mobile phase was prepared daily, filtered, and sonicated before use. The initial mobile phase was eluent A. A linear gradient was started from the initial conditions leading in 30 min to the final conditions containing eluent A and eluent B (1 : 1). Then the mobile phase was linearly returned to the initial condition within 5 min. After 10 min, the next sample was injected. The analysis of ODN was realized at 256 nm

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wavelength, and the determination of ODN–DOX conjugate was realized at 256 nm and 480 nm wavelengths.

Preparation of Standard Solutions

The conjugate and ODN standard solution were prepared by dissolving 30 OD of ODN and ODN–DOX conjugate, respectively, in PBS (PH 7.4) and, then, diluted to the range of 2–600 μg/mL using PBS buffer. The solutions were storage at −20°C, and were defrosted before used. For calibration in plasma, an aliquot of ODN or ODN–DOX conjugate solution with known concentration was mixed with 100 μL of the defrosted plasma, and then diluted with PBS containing 30 IU/mL heparin to 200 μL of total volumes. The mixture was centrifuged for 10 min at 14,000 rpm at 4°C, and then the supernatant was collected and stored at −20°C until used. The calibration curve for HPLC analysis was accomplished by plotting the ratio of the peak area of ODN or ODN–DOX conjugate to their concentration.

Study of the Stability of Oligodeoxynucleotide–Doxorubicin Conjugate In Vitro

The solution of ODN–DOX conjugate (20 μL, 1 μg/μL) and ODN (20 μL, 1 μg/μL) were incubated at 37°C in 10% activated fetal calf serum (80 μL), respectively. After incubation for various regular times, 5 μL of the mixture was drawn and mixed with 45 μL dilution buffer and the mixtures were heated at 70°C for 5 min, and then 20 μL was injected as a sample.

Pharmacokinetic Studies In Vivo

ODN and ODN–DOX conjugate were dissolved in physiological saline (0.9% NaCl). Animals (Male SD rats) were dosed via a single bolus i.v. injection into a tail vein at a dose of 30 mg/kg of ODN or 30 mg/kg of ODN–DOX conjugate. Doses were based on the pretreatment body weight and rounded to the nearest 10 μL. After injection, 100 μL blood was collected from the tail vein of each mouse by tail amputation from 1 to 48 hours, and then diluted to double volume with PBS (pH 7.4), which contained at least 30 IU/mL heparin for preventing coagulation. Each blood sample was centrifuged at 14,000 g/min for 10 min at 4°C, supernatant was then collected and stored at −80°C until analyzed.

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RESULTS AND DISCUSSION

A typical chromatogram of ODN and OAN–DOX conjugate in PBS and plasma are shown as Fig. 1. Both compounds appear as one peak with good baseline resolution. ODN and ODN–DOX conjugate were well separated on the RP column with the retention times at 9.16, 17.67 min, respectively, at the chromatographic conditions, with a flow rate of 1.0 mL/min. The peak of ODN–DOX conjugate appears at both 254 and 480 nm (the maximum absorption of DOX) with the same retention time, while ODN has no peak at 480 nm. There are no extraneous peaks near the peaks of ODN and ODN–DOX conjugate in chromatograms obtained for plasma samples.

Analytical parameters for the peak area ratio of varying amounts of two compounds in PBS and in plasma are shown as Table 1. The injection volume was 50 μL. Standard curves of ODN and ODN–DOX conjugate in PBS were linear over 0.1–660 and 0.1–820 μg/mL, respectively, with coefficient of determination (r^2) values of greater than 0.99. In plasma, the conjugate was

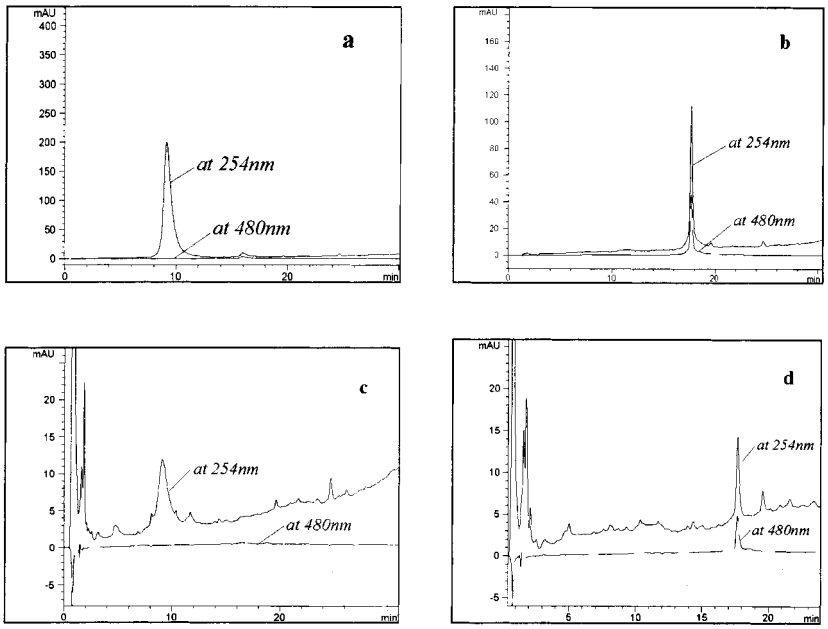

Figure 1. Typical RP-HPLC chromatograms of standard solution of ODN or ODN–DOX conjugate in the PBS or plasma with the chromatographic conditions: (a) ODN (155.06 μg/mL) in PBS; (b) ODN–DOX conjugate (112.75 μg/mL) in PBS; (c) ODN (6.45 μg/mL) in plasma; (d) ODN–DOX conjugate (12.52 μg/mL) in plasma.

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Table 1. Analytical parameters for the determination of ODN and ODN–DOX conjugate in PBS or in plasma.

Compound	ODN at 254 nm	Conjugate in PBS		Conjugate in plasma	
		254 nm	480 nm	254 nm	480 nm
Linearity range (μg/mL)	0.1–780	0.1–890	0.1–830	0.20–460	0.15–510
r^2*	0.9921	0.9987	0.9976	0.9236	0.9301
LOD* (ng/mL)	1.65	1.02	1.21	3.98	2.62
LOQ* (ng/mL)	5.51	3.40	4.03	13.27	8.73

Note: LOD*, The limit of detection; LOQ*, The limit of quantitation; r^2*, Coefficient of determination; Three samples were processed at each concentration.

linear over 0.15–510 μg/mL with r^2 values of greater than 0.92. However, for ODN in plasma, the values present non-linearity (date not shown) because of degradation. The limit of detection (LOD) was determined as the signal corresponding to 3 times the peak-to-peak noise. The limit of quantitation (LOQ) was calculated as the signal corresponding to 10 times the peak-to-peak noise.

Precision and reproducibility were examined within-day and between-day in PBS and in plasma, respectively. The results are shown as Table 2. It was concluded that, for ODN–DOX conjugate, there was no significant difference for the assay tested within-day and between-day, both in PBS and in plasma. But, for ODN there was a remarkable decrease for the tested between-day in the plasma sample. It may be caused by the degradation of ODN which is endogenous in plasma.

The recovery experiments were carried out by adding known amounts of compounds to the pre-analysis plasma samples. The results are presented in Table 3. For ODN–DOX conjugate, the recovery was close to 100%, both in PBS and in plasma. However, for the ODN plasma sample, it was close to 100% in PBS and 68% in plasma. The results indicate that the method is reliable for analysis of ODN–DOX conjugate, both in PBS and in plasma.

The Stability In Vitro

Figure 2 shows the quantitative results obtained by RP-HPLC analyses. When incubated in 10% activated fetal calf serum for 24 hours at 37°C, 15.8% of the conjugate was degraded. As a comparison, however, 97.2% of the

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Table 2. Results of within-day and between-day precision of ODN and ODN–DOX conjugate in PBS or plasma.

Compound	Theoretical concentration (μg/mL)	Within-day measured concentration (μg/mL)		Between-day measured concentration (μg/mL)	
		Mean	RSD (%)	Mean	RSD (%)
ODN in PBS	3.10	2.93	0.86	2.89	1.26
	31.01	29.3	0.77	28.92	1.38
ODN in plasma	3.10	2.26	1.93	1.01	2.68
	31.01	26.5	1.67	20.32	2.87
Conjugate in PBS	4.75	4.56	0.62	4.51	0.81
	4.75	4.59[a]	0.79[a]	4.49[a]	0.74[a]
	42.75	41.25	0.64	40.85	0.78
	42.75	41.17[a]	0.82[a]	39.98[a]	0.76[a]
Conjugate in plasma	4.75	4.53	0.85	4.43	0.67
	4.75	4.49[a]	0.78[a]	4.42[a]	0.61[a]
	42.75	40.75	0.94	40.14	0.71
	42.75	40.56[a]	0.66[a]	39.15[a]	0.54[a]

Three samples were processed at each concentration.
[a]Each value was detected at 480 nm.

control ODN was degraded after the same time incubation. The same overall result was obtained with 20% polyacrylamide gel containing 7 M urea by electrophoresis analysis (figure not shown). As expected, these results proved that attachment of the DOX at the 3′-end of the ODN protect it against degradation by nuclease.

Pharmacokinetic Studies

Figure 3 illustrates the total plasma concentration-time profile of ODN and ODN–DOX conjugate in BD rats, following i.v. via the tail vein with a dose of 30 mg/kg. Following a single injection, ODN and ODN–DOX conjugate were detected in plasma using the proposed method at 1 hour later. The elimination half-life of ODN is about 2 hours, whereas, the conjugate's is about 8 hours. The reason that the half-life of the conjugate is 4 times longer than ODN may be due to the strong stability of the conjugate.

Table 3. Results of the recovery analysis of ODN and ODN–DOX conjugate in PBS or in plasma.

Compound	Added concentration (μg/mL)	Recovery (μg)	Recovery (%)	RSD (%) of recovery
ODN in PBS	3.28	3.08	94.0	0.67
	19.68	18.42	93.6	0.46
ODN in plasma	3.28	2.21	68.1	1.52
	19.68	15.65	79.5	0.86
Conjugate	3.07	2.93	95.4	0.23
in PBS	3.07	2.91[a]	94.8[a]	0.35[a]
	18.42	17.49	95.0	0.49
	18.42	17.54[a]	95.2[a]	0.48[a]
Conjugate	3.07	2.91	94.8	0.43
in plasma	3.07	2.87[a]	93.5[a]	0.46[a]
	18.42	17.44	94.7	0.43
	18.42	17.34[a]	94.1[a]	0.57[a]

Each value was the mean of three experiments.
[a]The value was detected at 480 nm.

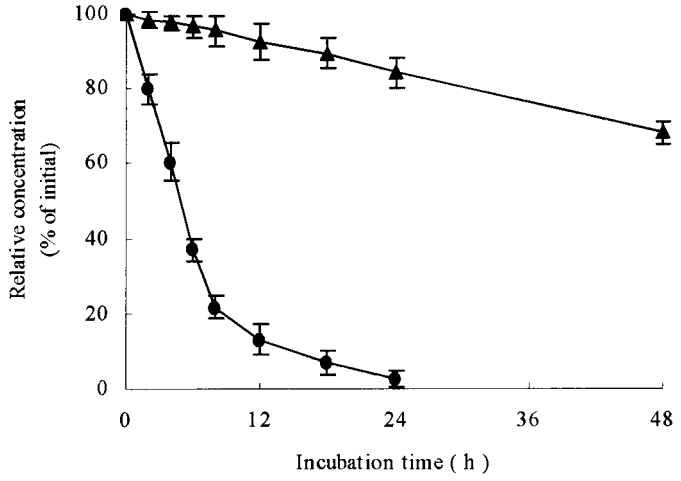

Figure 2. Stability of AS ODN and AS ODN–DOX conjugate in 10% fetal calf serum at 37°C in the dark. Determination by HPLC at 254 nm using a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate, pH 7.0, with a flow rate of 1 mL/min. *Key:* (●): AS ODN; (▲): AS ODN–DOX conjugate.

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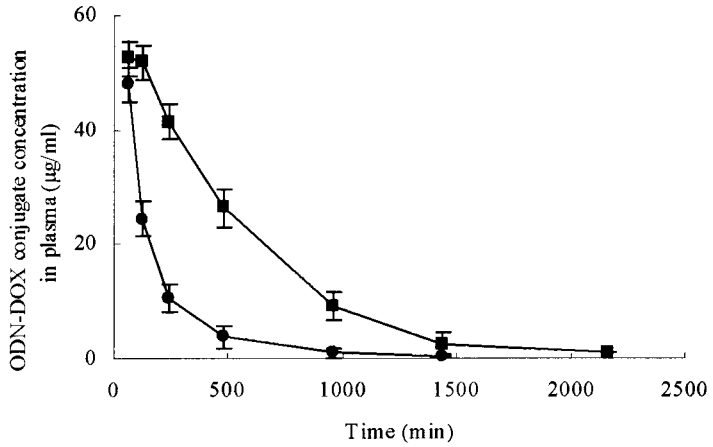

Figure 3. ODN and ODN–DOX conjugate concentration-time profile measured by RP-HPLC in plasma following i.v. doses of 30 mg/kg, respectively, to healthy SD rats. *Key:* (●): ODN; (■): ODN–DOX conjugate.

CONCLUSION

An RP-HPLC assay has been developed and validated to determine levels of ODN–DOX conjugate in animal (healthy SD rats) plasma. The peak of the ODN–DOX conjugate can be identified by its retention time at characteristic absorption wavelengths of both ODN and DOX using two-fold detection. Also there are no extraneous peaks in chromatograms obtained in the chromatography conditions used. The method developed appears to be accurate, precise, sensitive, reliable, and reproducible without complicated sample treatment, and can be used for pharmacokinetic study of the conjugate.

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