

©2004, Acta Pharmacologica Sinica  
Chinese Pharmacological Society  
Shanghai Institute of Materia Medica  
Chinese Academy of Sciences  
<http://www.ChinaPhar.com>

# Detection and quantitation of PS20, a phosphorothioate oligodeoxynucleotides in monkey plasma<sup>1</sup>

Ming-mei SHANG, Hai-feng SONG<sup>2</sup>, Xiu-wen LIU, Zhong-ming TANG

*Department of Pharmacology and Toxicology, the Institute of Radiation Medicine,  
Academy of Military Medical Sciences, Beijing 100850, China*

**KEY WORDS** antisense oligodeoxyribonucleotides; thionucleotides; rhesus monkey; plasma; solid-phase extraction; capillary electrophoresis; non-gel sieving; internal standard

## ABSTRACT

**AIM:** To establish the method for quantitation of the phosphorothioate oligodeoxynucleotides (S-ODNs) in plasma. **METHODS:** Two solid-phase extraction columns combined with a strong anion-exchange column were utilized to remove proteins and lipids in plasma, and the salts were removed by a reverse-phase column followed by dialysis with a 2500 Da-cutoff membrane. The concentration of the tested S-ODNs, PS20, and its metabolites extracted from the plasma were determined by the method of non-gel sieving capillary electrophoresis (NGCE) with diode array detection in the presence of internal standard (IS). **RESULTS:** The method was with good base number specificity. Relative standard deviation % of both intra and inter assay were all less than 10 %, and the total mean recovery was about 91 %. The methodology was successfully used to determine the PK behavior of an anti-tumor antisense S-ODNs in monkeys and identify the metabolites with single base difference. **CONCLUSION:** The combined method of solid-phase extraction and NGCE could be used to study the pharmacokinetics of S-ODNs, and the main parameters of the methodology met the requirement of PK study.

## INTRODUCTION

The development of antisense compounds as therapeutic agent is moving forward rapidly. In 1998, the FDA approval of the Vitravene (ISIS 2922) highlighted the progress made in moving antisense oligonucleotides from the laboratory to the market place<sup>[1]</sup>. However, questions continually arise regarding the pharmacokinetics and the toxicity of these novel therapeutic agents<sup>[2]</sup>. As a new class of therapeutic compounds, oligonucle-

otides lack established bio-analytical method, and therefore, pose unique analytical challenges. Early studies relied on ion-exchange separation of the radio labeled oligodeoxy-nucleotides followed by scintillation counting for detection<sup>[3]</sup> or hybridization technique<sup>[4,5]</sup> for quantitation of drug. Although these methods gave good approximations of oligodeoxynucleotides concentrations, they did not provide separation of the parent oligonucleotide from the metabolites truncated by loss of a single nucleotide unit. Since 1990s, with the maturation of the capillary electrophoresis technology, some investigators had successfully used capillary gel electrophoresis (CGE) to separate S-oligodeoxynucleotides (ODN)s up to approximately 25-nucleotide-long<sup>[6-8]</sup>. An internal standard (IS) was usually used to normalize for the bias caused by electro

<sup>1</sup> Project supported by the National Natural Science Foundation of China (No 39870878 and 39930180).

<sup>2</sup> Correspondence to Assoc Prof Hai-feng SONG.  
Phn/Fax 86-10-6693-1230. E-mail songhf@nic.bmi.ac.cn  
Received 2003-06-05 Accepted 2004-02-25

kinetic injection. Except CGE, the non-radio-labeled method for quantitation of the S-ODNs was very limited. In this paper, a novel non-radio-labeled method based on the technology of non-gel sieving capillary electrophoresis was established and validated on the purpose of S-ODNs quantitation.

## MATERIALS AND METHODS

**Materials** Desalting filters with pore size of 0.025  $\mu\text{m}$  were purchased from Millipore (Millipore, USA). Strong anion-phase solid-phase extraction cartridges (SAX SPE, 100 mg) and isolate reverse-phase solid-phase extraction cartridges (C-18 SPE, 100 mg) were both provided by Phenomenex (Phenomenex, USA). The ssDNA 100-R kit was obtained from Beckman Coulter (Beckman Coulter, USA) which contained a coated capillary [65 cm $\times$ 75  $\mu\text{m}$  (ID)]. The N<sub>2</sub> with the purity of 99.5 % was the product of Beijing Pulekesy Practical Gas Co Ltd (Beijing, China). All other reagents were of analytical grade and purchased from commercial sources.

The tested S-ODNs, named PS20, was provided by Chengdu Di'ao Pharmaceutical Company (Chengdu, China), and other two S-ODNs which were set as the IS were synthesized by Sangon Biotechnology Co Ltd (Shanghai, China). The sequences of all S-ODNs used in the study were included in Tab 1. Before use, all S-ODNs were dissolved in distilled water (dH<sub>2</sub>O) and the concentrations were determined by spectrophotometer.

**Tab 1. The sequence of S-ODNs involved in the study.**

S-ODNs	Sequence 5'→3'
PS20	AGGGTCAGCTGCGTCGACAT
T27	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
K1470	AGGAGTGCAGCTGCGTCAAG
K1471	TCAAATGGAGGCTGCCCGGC

**Apparatus** The Simplicity185 water purification system was from Millipore, USA. The spectrophotometer was the DU<sup>®</sup> 640 Nucleic Acid and Protein analyzer (Beckman Coulter, USA). The P/ACE MDQ DNA System was purchased from Beckman Coulter, USA. The ultrasound instrument came from ELMA, Germany. The centrifugal apparatus was purchased from Beijing

Medical Centrifuge Machine Factory, China.

**Animals** Rhesus monkeys were supplied by the animal Raising Center of Academy of Military Medical Sciences (Grade I, Certificate BDW95002), weighed 3.5 $\pm$ 1.0 kg. The animals were individually housed in stainless-steel cages, and were fed with standard diet. Water was supplied *ad libitum*.

**Blood collection** Blood was collected with edetic acid-treated tubes and freshly drawn whole blood was mixed with sodium citrate (0.109 mol/L) at the ratio of 9:1. Then samples were centrifuged immediately at 750 $\times$ g for 10 min. Plasma was pipetted and stored at -20 °C before extraction.

**Plasma extraction procedure** Before extraction, the plasma was quickly thawed, then the known concentration of PS20 and IS (T27, which was recommended previously<sup>[9]</sup>) were added and kept at 4 °C until analysis. The samples were incubated at room temperature briefly (less than 5 min) before loading onto the SAX SPE. Firstly, the SAX SPE columns were pre-equilibrated with 1 mL acetonitrile (ACN), followed by 1 mL dH<sub>2</sub>O wash and 3 mL of the loading/running buffer (Tris-HCl 50 mmol/L, pH 9.0, KCl 0.5 mol/L, 20 % ACN) wash. Then the samples were loaded onto the SAX-SPE column after equilibrating with 3 mL loading/running buffer. After a 1-mL dH<sub>2</sub>O wash, the S-ODNs were eluted from the SAX SPE column with 1 mL of the SAX elution buffer (Tris-HCl 50 mmol/L, pH 9.0, KCl 0.5 mol/L, NaBr 1.0 mol/L, 30 % ACN). The collected elute was stored at 4 °C until loading onto the C-18 SPE columns. The C-18 SPE columns were pre-equilibrated with 1 mL ACN, 1 mL dH<sub>2</sub>O, and 3 mL SAX dilution buffer (Tris-HCl 50 mmol/L, pH 9.0, KCl 50 mmol/L, NaBr 100 mmol/L) successively. The SAX elute was loaded onto the C-18 SPE column and washed with 5 mL dH<sub>2</sub>O. S-ODNs were eluted using at least 3 mL of freshly prepared 40 % ACN. Then the samples were dried by flowing N<sub>2</sub> and dissolved in 60  $\mu\text{L}$  dH<sub>2</sub>O followed by a further desalt procedure, in which the samples were firstly dropped on a Millipore 0.025- $\mu\text{m}$  VS membrane floating on dH<sub>2</sub>O for 30 min and then bubbles were eliminated by supersonic wave and centrifugation.

**Non-gel sieving capillary electrophoresis analysis** NGCE separations was accomplished with the MDQ DNA System using a 27-cm-long column with the effective separation length of 20 cm. Samples were electrokinetically injected at 10 kV for a duration of between 5 and 40 s. The length of time and voltage necessary to

produce an adequate signal depends on the concentration of the extracted sample and the amount of salt in the samples. Separation was achieved by operating at 25 °C at the operated voltage of 18 kV.

**Data procession and analysis** Area under the curve for S-ODNs peak was determined by the Beckman System 32Karat software. Integration parameters were the peak area threshold of 100 area units and the peak width of 0.01 min. The concentration of tested S-ODNs was determined by IS. The ratio of any two peaks at the UV 255 nm is proportional to the ratio of the areas divided under the same separation condition. The quotient of the area divided by the migration time for any particular oligodeoxynucleotide is referred to as the corrected area. The corrected area of the analyte of interest divided by the corrected area of the IS is referred to as the normalized area<sup>[10]</sup>. This value was used when determining the linearity of the assay. The formula used to calculate the concentration of tested S-ODNs and its metabolites was as follows:

$$C_1=C_2 (E_2/E_1)[(A_1/T_1)/(A_2/T_2)].$$

Here,  $C$  is the concentration,  $E$  represents the molar extinction coefficient,  $A$  is the area of the peak,  $T$  represents the migration time, and the subscript represents the samples respectively<sup>[3]</sup>. Finally, version 5.0 of MicroCal Origin software was used to fit and plot the data finally. Statistical inferring was obtained by means of  $t$ -test.

## RESULTS

**Validation of the methodology** According to the strategy in validation of the bio-analytical method of FDA, the specificity, LOQ, linearity, accuracy and precision of the method for quantitation S-ODNs was evaluated using solid-phase extraction combined with the NGCE in the presence of IS, which were the main parameters used to describe the bio-analytical method.

At first, the electrophoretic behavior of the PS20 and IS were inspected. Results suggested that the IS could be separated completely from the PS20 (Fig 1). Furthermore, three S-ODNs (K1470, K1471, and PS 20) with the same length (20-mer) but different base composition were separated. The results demonstrated that NGCE could hardly identify the composition difference, the peak of the mixture of them three seemed to be superposed (Fig 2).

The dose of antisense S-ODNs was very low, and the concentration of the PS20 was estimated to range from 0.5 mg/L to 10 mg/L after investigating lots of

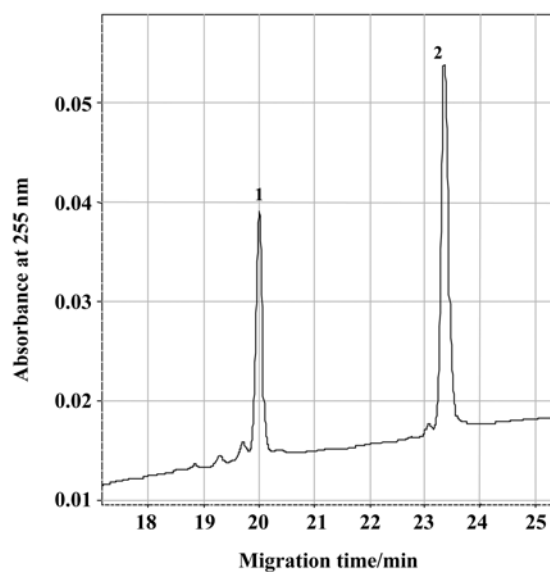


Fig 1. The PS20 and T27 were separated from the baseline. Peak 1 referred to the PS20, and the peak 2 referred to the T27. Both the concentrations were 10 mg/L.

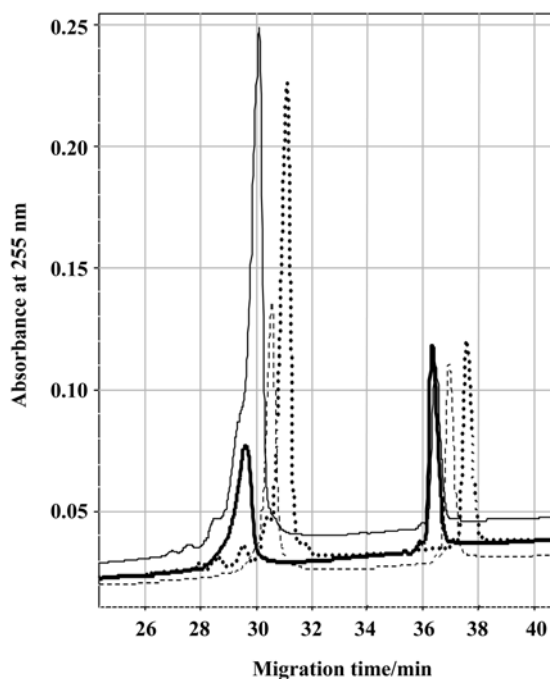


Fig 2. Electropherograms for mixture of three S-ODNs with the same base number and the IS, and the mixture of each oligomer and IS. The concentrations of IS were all spiked at 10 mg/L. ....: the mixture of K1471 and the IS; ----: the mixture of K1470 and the IS; —: total mixture including PS20, K1470, K1471, and the IS; -·-: the mixture of PS20 and the IS.

issues<sup>[11]</sup>. The concentration of the IS kept on 10 mg/L. The experiments were finished according to the procedure described above and each sample was extracted

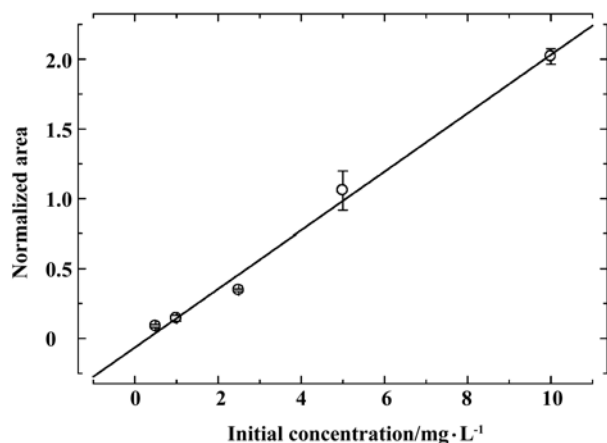


Fig 3. Standard curve for the extracted samples of the normalized area vs the initiation concentration of the PS20.  $n=5$ . Mean $\pm$ SD.  $r=0.996$ .  $P<0.01$ .

three times. The curve was plotted as the initial concentrations of PS20 versus the normalized area (Fig 3). The curve was linear with the square of the correlation coefficients ( $r^2$ ) of 0.992.

The reproducibility, accuracy and precision of the quantitation were determined by comparing the samples before and after extraction. The concentrated mixture of PS20 and IS was diluted by distilled water and rhesus monkey plasma respectively until the final concentrations were 10 mg/L. The water-solution sample was injected directly six times and the concentration of the PS20 was calculated by the IS. Then the plasma sample was extracted for six times and the concentration of PS20 was calculated by the IS according to the formula shown above. Then the two calculated concentrations were compared. The relative standard deviation % of the sample without extraction was 9.5 %, while the relative standard deviation % of the plasma sample after six times extraction was 9.6 %. The relative mean recovery of the PS20 was 91 % $\pm$ 10 %, which met the requirement of the pharmacokinetics study.

The reproducibility of the relative migration time was also assayed. Strictly speaking, the reproducibility of the method included two parts: one was the reproducibility of the quantitation and another was the reproducibility of the relative migration time. The RSD % of the relative migration time of the sample 0.5, 2.5, and 10 mg/L after the six times extraction was 1.6 %, 2.4 %, and 4.3 %, respectively.

**Analysis of the plasma samples in rhesus monkey** Electropherograms of starting material and plasma

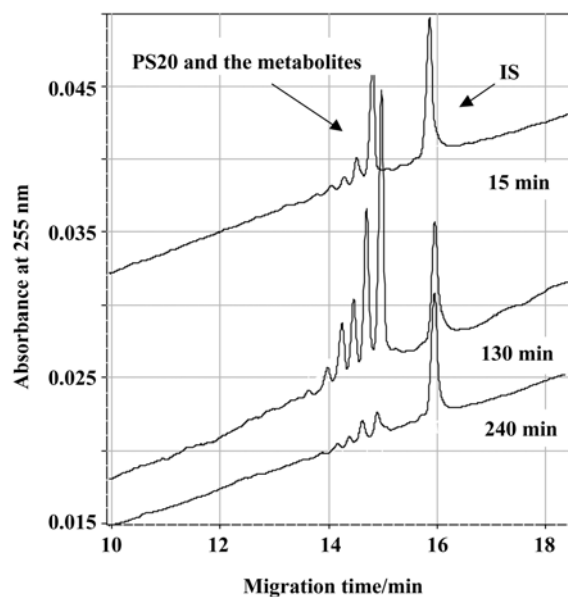


Fig 4. NGCE profiles of plasma taken at 15, 130, and 240 min following intravenous infusion of PS20 5 mg/kg for 2 h in rhesus monkeys. The plasma samples spiked with the IS up to concentration of 10 mg/L immediately before extraction. The peaks before PS20 were  $n-1$ ,  $n-2$ ,  $n-3$ , and  $n-4$  metabolites of PS20, which behaved as an administration time-related change.

extracts during and after a 2-h infusion of 5.0 mg/kg PS20 into healthy rhesus monkey subjects illustrated the typical pattern of metabolism (Fig 4), which showed the electrophoresis behavior of the extracts from the plasma taken at 15, 130, and 240 min following intravenous infusion administration. The peaks before the PS20 peak were the metabolites by loss of a single nucleotide unit in turn<sup>[12]</sup>. We can identify the  $n$ ,  $n-1$ ,  $n-2$ ,  $n-3$ , and  $n-4$  oligomers clearly according to the relative migration time. The results made clear that the concentration of the PS20 increased gradually during the intravenous administration and reached the mean peak concentration ( $C_{max}$ ) immediately at the end of the infusion. After an intravenous infusion, the full-length PS20 decreased while the shortened metabolite increased. The concentration-time curve of the PS20 and the metabolites were shown (Fig 5). The data demonstrated that the metabolites of the PS20 appeared rapidly after the initiation of the infusion, and the parent compound was almost always present even after four half-lives.

## DISCUSSION

In the study, a combined method comprised of

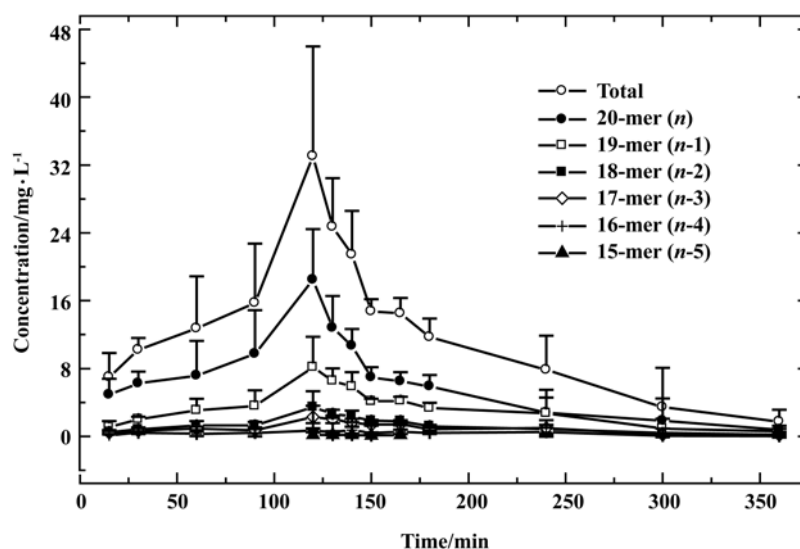


Fig 5. Plasma concentration of PS20 and its metabolites following intravenous infusion of PS20 5 mg/kg for 2 h in rhesus monkeys.  $n=3$ . Mean $\pm$ SD.

sequential anion-exchange, C-18 SPE, and a membrane-desalting step was utilized to extract the S-ODNs from the plasma samples, and the technology of NGCE was employed to determine the concentration of the extracted S-ODNs. Methodology validation study showed that the extraction did not affect the normalized area, the relative recovery, reproducibility, precision and the sensitivity of the method was suitable for the pharmacokinetics study of the antisense S-ODNs.

The CGE was the main separation mode of the capillary electrophoresis since later period of 1980's. The technology integrated the high efficiency of the gel electrophoresis and the high speed of capillary electrophoresis. The early researchers used CGE as separation method for the oligodeoxynucleotides and their metabolites, but it was very difficult to prepare and store filled capillary because of the fixed properties of the gel<sup>[13]</sup>. Besides, the short life of the capillary and the high cost limited application of CGE in this field. So researchers were engaged in looking for new capillary electrophoresis mode. Zhu *et al* firstly reported the technology of NGCE in 1989<sup>[14]</sup>. Since then more researchers preferred to use NGCE instead of traditional CGE, and NGCE was improved rapidly. In these days, the samples were able to be separated in NGCE through the total charges and the molecule weight of the molecule<sup>[15]</sup>. Compared with CGE, NGCE cost quite lower, the capillary was easy to be prepared and the sieving media could be exchanged conveniently. With comparable sensitivity and efficiency, NGCE was cer-

tainly superior to traditional CGE.

The reproducibility of all kinds of the capillary electrophoresis was not as ideal as expectation of researchers. To the best knowledge, there was very little report about the reproducibility of the capillary electrophoresis using coated capillary in literature. Too many factors such as temperature of the cartridge, quantity of the sample injected, separation conditions *etc* influenced the results so that the researchers could not control the capillary electrophoresis completely<sup>[15]</sup>. The condition must be optimized for obtaining the optimum results. Besides, many investigators calibrated the tested samples using IS. In fact, it is necessary to calibrate the integration area of the peaks in capillary electrophoresis indeed, especially in NGCE because there are more factors influencing the behavior of the S-ODNs than capillary zone electrophoresis (CZE), CGE or so. Furthermore, the volume of the loaded gel affected the inside environment of the capillary. If the gel loaded into the capillary was not homogeneous, the electric field would be heterogeneous. Therefore the behavior of the oligodeoxynucleotide would change with the electric field. And, the microenvironment and the electric field inside the capillary changed after every run. When the oligodeoxynucleotides were injected into the capillary, they migrated in the electric field because of the charges carried. So the order of the runs of the sample after loading gel influenced the results too. Finally, it was impossible to require the capillary and gel from different batches identical.

Leeds *et al*<sup>[3]</sup> reported that the tested S-ODNs and

the IS were able to be proportionally extracted, but our results showed that the relative recovery of the method might be related to the conformational structure of the IS and the T27, the traditionally used IS in previous studies, might not be the optimal IS for quantitation of all S-ODNs. An individualized IS with the composition similar to tested S-ODNs was used to improve the recovery (data not shown). Of course, the optimization of the IS still needs further and deeper studies.

**ACKNOWLEDGEMENTS** This study was funded in part by Chengdu Di'ao Pharmaceutical Company, Chengdu, China. We acknowledge the excellent technical assistance of Mrs Bao-zhen ZHU, Mrs Ling-zhen LI, and Mr Yang ZHANG.

## REFERENCES

- 1 Roehr B. Formivirsen approved for CMV retinitis. *J Int Assoc Physicians AIDS Care* 1998; 4: 14-6.
- 2 Levin AA. A review of issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* 1999; 1489: 69-84.
- 3 Leeds JM, Graham MJ, Truong L, Cummins LL. Quantitation of phosphorothioate oligonucleotides in human plasma. *Anal Biochem* 1996; 235: 36-43.
- 4 Temsamani J, Kubert M, Agrawal S. A rapid method for quantitation of oligodeoxynucleotide phosphorothioate in biological fluids and tissues. *Anal Biochem* 1993; 215: 54-8.
- 5 Turano B, Humphreys T. Quantitative determination of phosphorothioate-modified oligodeoxyribonucleotide persistence. *Biol Techniques* 1994; 17: 266-7.
- 6 DeDionisio L. Capillary gel electrophoresis and the analysis of DNA phosphorothioates. *J Chromatogr A* 1993; 652: 101-8.
- 7 Cohen AS, Vilenchik M, Dudley JL, Gemborys MW, Bourque AJ. High-performance liquid chromatography and capillary gel electrophoresis as applied to antisense DNA. *J Chromatogr A* 1993; 638: 293-301.
- 8 Phillips JA, Craig SJ, Bayley D, Christian RA, Geary R, Nicklin PL. Pharmacokinetics, metabolism, and elimination of a 20-mer-phosphorothioate oligonucleotide (CGP 69846A) after intravenous and subcutaneous administration. *Biochem Pharmacol* 1997; 54: 657-68.
- 9 Adjei AA, Dy GK, Erlichman C, Reid JM, Sloan JA, Pitot HC, *et al*. A phase I trial of ISIS 2503, an antisense inhibitor of H-ras, in combination with gemcitabine in patients with advanced cancer. *Clin Cancer Res* 2003; 9: 115-23.
- 10 Srivatsa GS, Batt M, Schuette J, Ronald H, Carlson RH, Fitchett J, *et al*. Quantitative capillary gel electrophoresis assay of phosphorothioate oligonucleotides in pharmaceutical formulations. *J Chromatogr A* 1994; 680: 469-77.
- 11 Bellon L, Maloney L, Zinnen SP, Sandberg JA, Johnson KE. Quantitative determination of a chemically modified hammerhead ribozyme in blood plasma using 96-well solid-phase extraction coupled with high-performance liquid chromatography or capillary gel electrophoresis. *Anal Biochem* 2000; 283: 228-40.
- 12 Wang YC, Xiong Y, Zeng ZR, Cheng JK, Shen P. Separation of DNA fragments from halobacterium by capillary electrophoresis with non-gel sieving media. *Chin J Chromatogr* 2001; 19: 439-42.
- 13 Epperson JD, Dodge J, Rill RL, Greenbaum NL. Analysis of oligonucleotides and unincorporated nucleotides from *in vitro* transcription by capillary electrophoresis in Pluronic F127 gels. *Electrophoresis* 2001; 22: 771-8.
- 14 Zhu MD, Hansen D, Brurd S. Factors affecting free zone electrophoresis and isoelectric focusing in capillary electrophoresis. *J Chromatogr* 1989; 480: 311-5.
- 15 An DK. Selected topics on modern pharmaceutical analysis. 1st ed. Beijing: Chinese Medical and Technological Publishers; 2001.