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Development and validation of a high performance liquid chromatography method for oligodeoxynucleotides determination in a novel coagel-based formulation

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ABSTRACT

The therapeutic benefit of phosphorothioate oligodeoxynucleotides (PS-ODN) containing immune stimulatory sequences has been demonstrated in animal models of cancer and infection. Several tools are available for the determination of these oligonucleotides in biological samples and pharmaceutical preparations, including UV spectroscopy, dye binding, isotopic tracing, capillary gel electrophoresis (CGE), hybridization-based enzyme-linked immunosorbent assay (ELISA), and chromatography techniques.

However, due to inter-assay variability and accuracy problems associated with the afore mentioned methods, we have developed and validated an isocratic high performance liquid chromatographic (HPLC) for analytical determination of PS-ODN containing unmethylated CpG motifs (CpG-ODN). Validation under Food and Drug Administration (FDA) guidelines of the analytical parameters include: linearity (r^2 0.9996), LOD (0.86 μg/ml) and LOQ (6.25 μg/ml), intra (0.19–3.37%) and inter-day precision (0.63–3.75%) expressed as relative standard deviation (RSD), and robustness parameters (less than 2.80%). Using this method, recoveries ranging from 89.9% to 99.9% were obtained. Thus, this method provides a simple, sensitive, precise and reproducible examination which can be readily adapted for the assessment of CpG-ODN in different pharmaceutical preparations.

1. Introduction

Phosphorothioate oligodeoxynucleotides (PS-ODN) containing unmethylated CpG motifs (CpG-ODN) have been developed as a novel first generation drug candidate for immune therapy in cancer and are currently in Phase I–III human clinical trials in non-small cell lung cancer, melanoma and cutaneous T cell lymphoma [1]. These are molecules stabilized by substituting non bridging oxygen with sulphur atoms to form a phosphorothioate backbone (Fig. 1) [2].

Since CpG-ODN are inherently prone to rapid degradation by nucleases which renders them inactive in the free form, the use of these chemical modifications have effectively reduced the sensitivity of ODNs to nuclease, prolonged circulation lifetime and improve their pharmacological/toxicological properties [3].

Nevertheless, the use of free CpG-ODN still faces several significant challenges including unfavorable pharmacokinetics, a lack of specificity for target cells and poor cellular uptake [4]. For this reason, numerous strategies have been developed in order to design and characterize novel carriers for these kinds of molecules [5].

Regarding this aspect, we have studied a variety of supramolecular aggregates formed by self-assembly of ascorbic acid derivatives, namely ascorbyl palmitate (ASC16) [6]. On cooling, this compound in water systems gives liquid crystals (called coagels) which, on cooling, becomes gels with lamellar structure. In previous works, we have explored such systems as carriers for CpG-ODN [7], specifically the coagel formed from ASC16 self-assembly (Coa-ASC16). Due to the complexity of the phase equilibrium observed in these lyotropic liquid crystalline systems [8], the evaluation of the biopharmaceutical performance of these novel formulations requires the development of adequate analytical methodologies.

Different bioanalytical methods to assay oligodeoxynucleotides with a PS backbone have been reported, such as UV spectroscopy,

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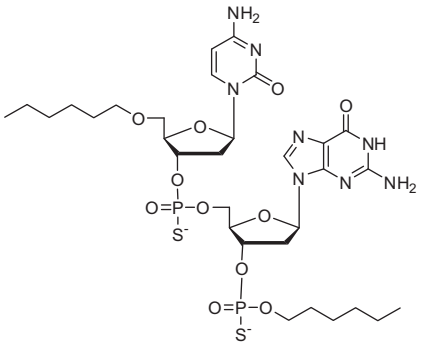

Fig. 1. Representative structure of CpG-motif showing functional groups on cytosine and guanine.

dye binding, isotopic tracing [9], capillary gel electrophoresis (CGE) [10], hybridization-based enzyme-linked immunosorbent assay (ELISA) [11], and chromatography techniques [12].

The accuracy of an absorbance measurement may be unreliable since it is based on the unconfirmed assumption that the DNA is pure and entirely in a double-stranded or single-stranded form. Dye-binding assays in general are subject to interferences and surfactants represent one of the most important. Postlabeling is limited both by its reliance on a radioisotope, and on the assumption, which is not convenient to test, that the yield of radio enzymatic step is 100% [13].

Although segregating techniques, including CGE were developed to separate PS-ODN from their interfering metabolites, their efficacy was limited by the low reproducibility caused by endogenous interference as well as cumbersome sample preparation [14].

Regarding ELISA, they have high sensitivity and could be used in the terminal phase of pharmacokinetics assessment, but these methods cannot distinguish full-length PS-ODN from their metabolites, which may cause cross-hybridization and the overestimation of the parent drug [15].

One common problem in developing an HPLC method for quantitative PS-ODN determinations is the extensive sample clean-up required. More recently, one-step solid-phase extractions (SPE) have been described in conjunction with liquid chromatography-mass spectrometry (LC/MS), but a major limitation of these methods is the low extraction recovery.

Taking into account these considerations, the aim of this study was to develop and validate a fast, reliable, selective and economic HPLC method able to quantify minimal CpG-ODN concentrations in different pharmaceutical systems. This article also includes results concerning to the effect of assay conditions over the stability of CpG-ODN and the protective effect that coagel could exert on CpG-ODN stability against enzymatic activity of exonucleases.

2. Methods

2.1. Chemicals and reagents

The CpG-ODN (sequence 5′-TCCATGACGTTCCTGACGTT-3′, phosphorothioate backbone) was purchased from Operon Technologies Inc. (Alameda, California, USA) and 6-O-ascorbyl palmitate (ASC16) was purchased from Fluka Analytical (Milan, Italy). Buffer tris(hydroxymethyl)aminomethane (Tris-HCl) was obtained from Biopack® (Buenos Aires, Argentina). Acetonitrile (ACN), triethylamine (TEA) and acetic acid (AA) were all HPLC-grade and acquired from Sintorgan (Buenos Aires, Argentina). Purified water namely milli-Q water was obtained from Merck Millipore® (Billerica, Massachusetts, USA).

2.2. Equipment and software

The HPLC analysis was carried out in an Agilent Technologies Series 1100 apparatus (Waldbronn, Germany) equipped with a binary pump, an auto sampler tray with column compartments, and a UV-Vis detector (λ_{max} absorbance 261 nm). Instrumental control and chromatographic data acquisition were performed with Agilent ChemStation Rev. B.03.01 software (Waldbronn, Germany). A Mettler Toledo DG 115-SC (Columbus, Ohio, USA) equipped with a combined Ag/AgCl/glass electrode was used to measure the experimental pH of the solutions.

2.3. Chromatographic conditions

The analytical column was a reversed phase C18 (250 × 4.6 mm i.d., 5 μm particle size) Phenomenex TM® (Torrance, California, USA) protected by a Security Guard Phenomenex TM® precolumn (Torrance, California, USA). Elution was performed isocratically at 25 °C at a flow-rate of 0.7 mL min^{-1}. The mobile phase was filtered through a 0.45 μm pore size filter (Merck Millipore®, Billerica, Massachusetts, USA) and degassed by vacuum prior to use.

2.4. Mobile phase and standard solutions preparation

A stock solution of CpG-ODN was prepared by reconstitution of CpG-ODN in saline solution (NaCl 0.9%) to reach a final concentration of 1000 μg mL^{-1}. This solution was stored at −20 °C.

Standard solutions were prepared by diluting different aliquots of the stock solution in buffer Tris-HCl pH 7.2. Next, the samples were filtered through a 0.22 μm pore size filter (Merck Millipore®, Billerica, Massachusetts, USA), transferred into auto sampler vials and injected (20 μL) into the HPLC system.

The mobile phase was prepared by measuring 5.70 mL of AA and 13.90 mL of TEA and mixed with Milli-Q water to reach a final concentration of 0.1 M in triethylammonium acetate buffer (TEAA). Subsequently, the pH was adjusted to 7 with AA. The final composition of mobile phase was buffer: acetonitrile (50/50).

2.5. Preparation of CpG-ODN loaded coagels (Coa-ASC16)

The samples were prepared by mixing the components (Asc16, water, and CpG-ODN) in the appropriate proportions in closed glass tubes. The dispersions were heated up to 72 °C and then homogenized in an ultrasonic bath for 15 min at 72 °C and left to reach room temperature in small, hermetically closed plastic tubes and stored in darkness until measurement. Samples were prepared to reach a final concentration of 0.02% and 1% weight/weight (w/w) fractions of ASC16 and CpG-ODN, respectively.

2.6. Nuclease stability of CpG-ODN load into Coa-ASC16

In order to evaluate the effect induced by Coa-ASC16 on CpG-ODN stability, we performed a nuclease digestion assay. CpG-ODN or CpG-ODN load into coagels (CpG-ODN/Coa-ASC16) samples were exposed to a 3′-exonuclease I enzyme solution (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) prepared according to manufacturer specifications and incubated at 37 °C. The CpG-ODN remaining at each time point was quantified by the HPLC technique.

3. Results and discussion

3.1. Optimization of experimental conditions

In order to find the best composition of the mobile phase that allows the analysis of CpG-ODN alone and load into Coa-ASC16, each compound separately was injected in mobile phases at pH 7 containing TEAA buffer and ACN at the following ratio: 90/10; 80/20; 70/30; 60/40; 50/50; 40/60;30/70; 20/80; 90/10.

In the case of mobile phases with high polarity, e.g., 20/80 and 80/20, the overlapping of some signals was observed, whereas an intermediate polarity allowed the molecule of interest to be eluted with a convenient peak resolution. Following the criteria of maximum resolution – minimum analysis time, TEAA buffer: ACN (50/50) was selected as the most suitable mobile phase for the quantification of CpG-ODN.

In these experimental conditions, chromatographic parameters such as capacity factor (k), and resolution (R_s) were 2.3 and 4, respectively.

At flow rates greater than 1 mL min^{-1} the retention time for CpG-ODN was shorter than 1.5 min, which resulted in the superposition of peaks corresponding to test and solvent samples. On the other hand, flow rates smaller than 0.5 mL min^{-1} resulted in non-acceptable peak resolution. Hence, the flow rate was set at 0.7 mL min^{-1}. In the optimized conditions, acceptable retention time of around 2.6 min was observed for CpG-ODN.

Fig. 2 shows the chromatogram obtained after the injection of CpG-ODN loaded in Coa-ASC16 (prepared as described in Section 2.5.) and diluted with mobile phase prior to injection.

High resolution (R_s > 1.5) was obtained for CpG-ODN in spite of the complexity of the system (CpG-ODN + Coa-ASC16) and the non-specificity of the selected column for oligonucleotides quantifications.

In the method reported here, we have achieved comparable results in terms of chromatographic parameters in comparison to those reported using more complex and expensive techniques [16]. In addition, neither solid-phase extraction method nor oligonucleotides precipitation techniques were required. Moreover, the tailing factors for CpG-ODN were within an acceptable range since good peak symmetry and resolution were achieved.

3.2. Validation method

This methodology has been validated according to the FDA validation guidance [17] in terms of linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy, selectivity, recovery and robustness.

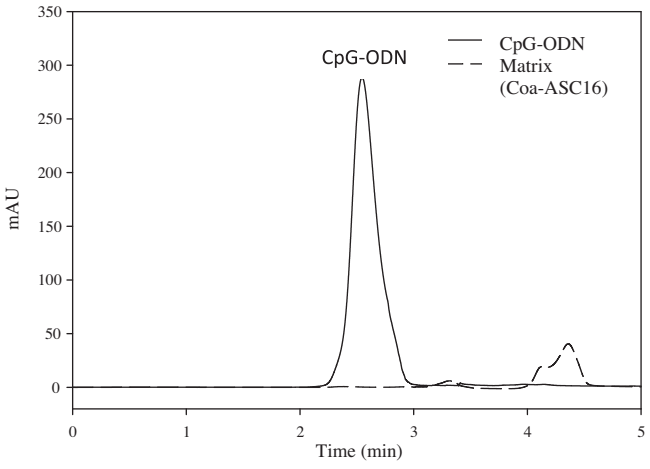

Fig. 2. Typical HPLC chromatogram of a standard sample preparation containing CpG-ODN loaded into Coa-ASC16. CpG-ODN retention time is 2.6 min. Refer to text for HPLC conditions.

3.2.1. Calibration parameters, limits of detection (LOD) and quantification (LOQ)

A series of standard curves were assayed over a concentration range of 6.25–375 μg mL^{-1}. Triplicates of seven points from three calibration curves were analysed every 3 days for 2 weeks. The data of correlation between peak areas versus drug concentration was statically processed by linear least square regression analysis. The lineal regression data for calibration plots (n = 3) showed a good linear relationship over the range of concentration evaluated. The coefficient of correlation was 0.9988, with slope and intercept values of 54161.33 ± 53.14 and 168.28 ± 6.90, respectively. No significant difference was observed in the slopes of standard curves (ANOVA p > 0.05).

The limit of quantification (LOQ) was considered as the lowest standard concentration in the analytical run affording accuracy and precision ⩽20% and in this method it was 6.25 μg mL^{-1}.

The limit of detection (LOD) was calculated with the 3 s criterion (three times the standard deviation of the lowest concentration solution included in the calibration divided by the slope of the calibration curve) [18] using a series of 7 solutions and its value was 0.866 ± 0.017 μg mL^{-1}.

3.2.2. Intra- and inter-day precision and accuracy

The accuracy and precision of the method were determined by triplicate analysis of sets of three quality control (QC) standards at concentrations of 250 μg mL^{-1} (QC-high), 125 μg mL^{-1} (QC-intermediate) and 50 μg mL^{-1} (QC-low). The intra-day precision was determined by injecting these three test solutions seven times on the same day. The inter-day precision determination involves the average of seven measurements of the intra-day precision values taken on ten days over a 2-month period.

Table 1 represents the results expressed as the percentage of the relative standard deviation and relative error (RSD, %) or coefficient of variation (CV) for the intra- and inter-day values. Reproducibility methods were acceptable since the CVs for the QC over the analytical range was <3.75% for intra- and inter-day precision assays. These results endorse the potential utility of the proposed method for the analysis of this compound in Coa-ASC16 formulations. Thus, the procedure developed can be used in the quality control, routine analyses and stability studies of CpG-ODN.

3.2.3. Recovery studies

In a new series of experiments, the QC-high and QC-low solutions (seven replicates for each standard) were spiked in Coa-ASC16 matrices, and the CpG-ODN amount previously diluted in mobile phase was measured. Standard solutions were processed

Table 1
Precision and accuracy of the HPLC assay for CpG-ODN in Coa-ASC16.

Nominal concentration (μg mL^{-1})	Calculated concentration (mean ± SD) (μg mL^{-1})	Precision R.S.D. (%)	Accuracy (%)
Intra-day evaluation			
QC-high-250	247.38±4.14	1.66	98.95
QC-intermediate-125	111.29±4.61	3.37	90.66
QC-low-50	46.89±0.01	0.19	93.78
Inter-day evaluation			
QC-high-250	248.45±6.88	2.75	99.38
QC-intermediate-125	111.06±4.70	3.75	90.36
QC-low-50	48.16±0.31	0.63	96.32

S.D.: standard deviation; n = 7 (number of replicates).

and analysed following the above-described procedure. Relative (analytical) recovery was calculated by comparing the concentration obtained from the standard solution with the actual added amounts. The data obtained shows satisfactory recoveries for CpG-ODN (First row, Table 2).

It is not mandatory to recover 100% of the analyte, but the extent of recovery of it should be consistent, precise, and reproducible.

Regarding this, the recovery of CpG-ODN was tested in samples conserved at room temperature for 5 h (normal run time for each batch) and in those frozen at −20 °C for 8 weeks. The samples were analysed immediately after the storage period. As it is shown in Table 2, the recovery results after the long-term and the short-term storage conditions were also satisfactory.

3.2.4. Robustness

The robustness of the method was examined by replicate injections ($n = 5$) of 50 μg mL^{-1} and 100 μg mL^{-1} standard solutions with slight modifications on the chromatographic parameters (buffer concentration, temperature, and flow rate). The R.S.D. (%) obtained after changing the retention time and peak area was calculated, being less than 2.80% (Table 3). In conclusion, variations in all the studied parameters had no significant effects on retention time or peak area, and the developed method proved to be robust for CpG-ODN quantifications.

3.3. Application of the HPLC method to nuclease digestion assays of CpG-ODN and CpG-ODN load into ascorbyl palmitate coagels

In order to challenge the performance of the HPLC technique, this method was used as an analytical tool to perform a stability assay of CpG-ODN against exonucleases. It is well known that nucleotide cleavage by exonucleases is the major degradation pathway of CpG-ODN and it occurs primarily from the 3′ end [19].

Table 2
Recovery (%) of CpG-ODN from Coa-ASC16 under different conditions.

	QC-high ($n = 7$)		QC-low ($n = 7$)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Normal conditions	97.63	3.90	99.90	1.70
−20 °C (8 weeks)	89.93	3.56	98.83	5.55
RT (5 h)	95.61	3.26	96.24	2.30

Table 3
Robustness evaluation of the HPLC method.

Chromatographic changes	CpG-ODN (100 μg mL^{-1})		CpG-ODN (50 μg mL^{-1})	
	t_R (min)	Area	t_R (min)	Area
Buffer TAA conc. (M)				
0.1	2.78	5796.36	2.84	2578.00
0.05	2.88	6004.21	2.81	2633.16
Mean	2.83	5900.29	2.83	2605.58
SD	0.07	146.97	0.02	39.00
RSD (%)	2.50	2.49	0.75	1.50
Temperature (°C)				
25	2.80	5629.22	2.80	2595.30
35	2.82	5410.42	2.86	2550.69
Mean	2.81	5519.82	2.83	2573.00
SD	0.01	154.71	0.04	31.54
RSD (%)	0.50	2.80	1.50	1.23
Flow rate (mL min^{-1})				
0.6	2.85	5300.00	2.85	2760.23
0.7	2.80	5500.00	2.83	2895.27
0.8	2.70	5480.00	2.73	2785.00
Mean	2.75	5490.00	2.78	2840.14
SD	0.08	110.15	0.06	71.89
RSD (%)	2.78	2.01	2.31	2.53

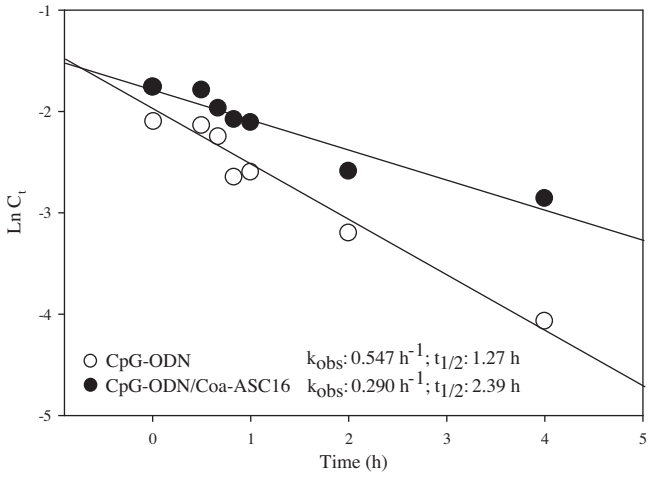

Fig. 3. Kinetics of pseudo-first order degradation of CpG-ODN and CpG-ODN/Coa-ASC16 in aqueous solutions of exonuclease enzyme.

As shown in Fig. 3, degradation of CpG-ODN follows a pseudo first order kinetics with a K_{obs} of 0.54 h^{-1} and a half-life of 1.27 h. When the same compound was formulated into the coagel, the half-life increased almost twice and it was observed a decrease in the degradation constant, which could result in an increase of its stability against degradation by exonucleases attributable to a protective effect of this carrier.

Based on these results, the enhancer effect of Coa-ASC16 on CpG-ODN adjuvant activity reported before [7] may be given, in part, by the effect of protection from degradation by nucleases. Thus, the use of Coa-ASC16 as strategy for delivering the CpG-ODN may therefore represent a valuable approach to protect them from nuclease degradation in future vaccination trials.

4. Conclusion

This paper describes a rapid and reproducible HPLC method which enables the determination of CpG-ODN in *in vitro* conditions. A simple and available chromatographic buffer system, with an usual C18 column was utilized for the validation process. The relatively short retention times facilitates the analysis of a large number of samples over a short period of time. Therefore, the HPLC method developed here shows good sensitivity and selectivity, and it is suitable for reliable determination of CpG-ODN.

Moreover, this method does not require complex procedures such as sample extraction and/or sample cleaning, and does not require large volumes of samples and solvents. Results indicate that the procedure described here is useful for the quantification of this compound loaded into Coa-ASC16 and the evaluation of its stability against exonuclease enzyme digestion.

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