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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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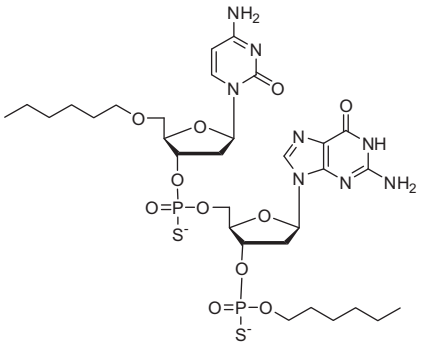

**Fig. 1.** Representative structure of CpG-motif showing functional groups on cytosine and guanine.

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 ($\lambda_{max}$ 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.