High-Performance Liquid Chromatography (HPLC) of Pharmaceutical Oligonucleotides in Non-Clinical Toxicology Research

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1 Introduction

Novel oligonucleotide-based drugs for the treatment of different diseases are widely pursued in the pharmaceutical industry. In non-clinical toxicology research, high performance liquid chromatography (HPLC) is the foremost analytical technology for quantitation and characterization of test pharmaceuticals in this category. Due to their large and complex structures and physical properties with respect to both non-covalent binding and surface adsorption, it is often challenging to establish sophisticated and reliable HPLC methods for the quantitation and characterization of oligonucleotides, as well as their related impurities, in test materials and dosage formulations. Oligonucleotides in test pharmaceuticals may consist of a composition of nucleotides with substitutions at the sugar's 2'- position and at the phosphate group (Figure 1). They may be in single or double stranded forms, and formulated in different dosage vehicles, including lipid nanoparticles for different dosing forms (injection solution, inhalation aerosol, et al). Using representative test pharmaceuticals, the chromatographic separation, online recovery of minor related impurities, and analysis of lipids in dosage nanoparticles are presented and discussed in this poster.

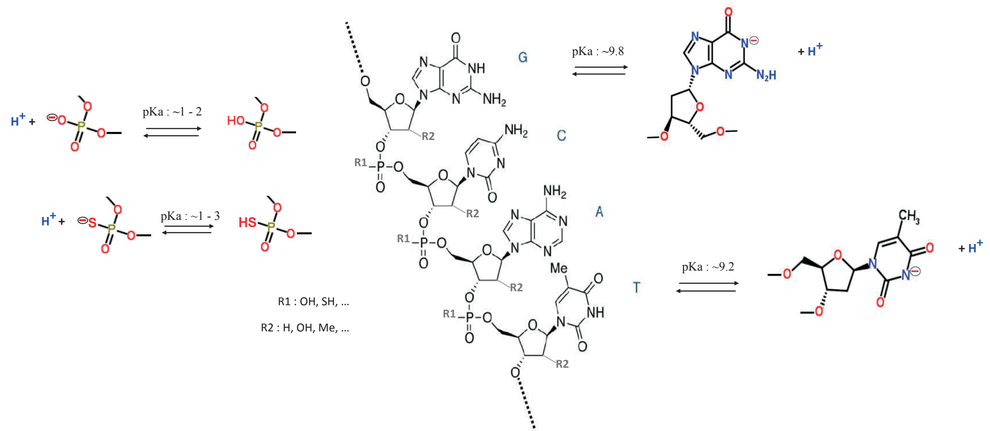

Figure 1. Common acidic dissociations of oligonucleotides in anion-exchange and ion-pairing reverse phase chromatography

2 Separation

Oligonucleotides are most commonly separated by anion-exchange or ion-pairing reverse phase chromatography, using the acidic dissociations of the phosphate and/or nucleobase (thymidine, quanosine, and uridine) groups (Figure 1). The acidic dissociations are strongly dependent on column temperature and solution pH, and by manipulation of these chromatographic parameters, resolution between oligonucleotides with similar molecular structure and size is demonstrated.

In general, the acidic dissociations increase with an increase in temperature (pKa1 – pKa2 = ΔH^o (1/T1 – 1/T2)/2.3R). However, there have not been any simple relationships observed between resolution and the influence of column temperature on these acidic dissociations in oligonucleotides. The column temperature for optimal resolution is most often found by trial-and-error. Figure 2A shows how the adjustment of column temperature influences the resolution of composite oligonucleotides (n = 4) in a test pharmaceutical, in ion-pairing reverse phase chromatography. Figure 2B compares the resolution of single stranded oligonucleotides (n = 3) in another test pharmaceutical, at low and high column temperatures, in anion-exchange chromatography.

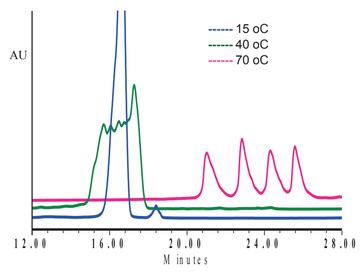

Figure 2A. Influence of column temperature on resolution of oligonucleotides in ion-pairing reverse phase chromatography (column: DNAPac RP, 3.0 x 100 mm, 5µm; counter ion: tetrabutyl- and tetraethyl-ammonium, pH 8.5)

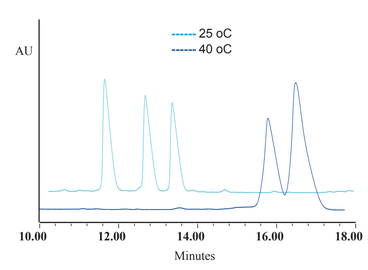

Figure 2B. Influence of column temperature on resolution of ologonucleotides in anion-exchange chromatography (column: DNA PAK PA200, 250 x 4.0 mm, 8u; elution agent: sodium bromide, pH 11.5)

In addition to column temperature, mobile phase pH can profoundly affect the acidic dissociations of thymidine, guanosine, and uridine in oligonucleotides, which will significantly impact the retention and separation. The pKa for these nucleosides is approximately 9.2 to 9.8 (Figure 1), thus mobile phase acidity for optimal resolution is usually found within the pH range of 8 to 11. Figure 3 presents a typical case of the separation of composite oligonucleotides (n = 3) in a test pharmaceutical using an anion-exchange column. Similar optimization of resolution by pH adjustment can be obtained in ion-pairing reverse phase chromatography, thanks to the availability of high pH columns.

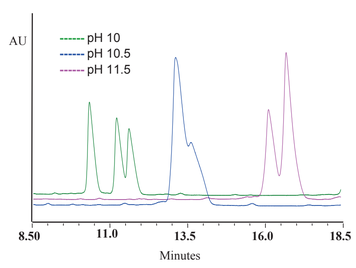

Figure 3. influence of mobile phase pH on resolution of oligonucleotides in anion-exchange chromatography (column: DNAPAK PA200, 250 x 4.0 mm, 8u; elution agent: sodium bromide)

3 Online recovery of minor impurities

Test pharmaceuticals in non-clinical safety testing require appropriate characterization, not only for purity of the material, but also for its related impurities. In the chromatographic process for oligonucleotides, full online recovery of related impurities is often challenging. In addition to losses resulting from their strong adsorption on metal surfaces, which can be minimized by adding modifiers such as surfactants to the mobile phase, the slow dynamics of oligonucleotides in the equilibration on the stationary phase often lead to poor online recovery. Figure 4 presents a typical example in which online recovery for minor impurities was dramatically improved at a lower flow rate. And although ultra high pressure systems are pushing LC to increasingly higher chromatographic speeds, it is often necessary to slow down the chromatographic process for large molecules like oligonucleotides and monoclonal antibodies.

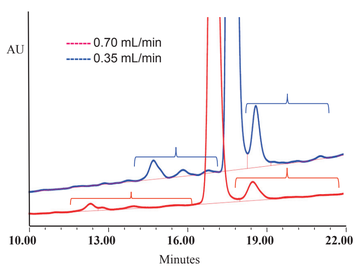

Figure 4. Impact of flow rate on online recovery of related impurities of an oligonucleotide (column: XBridge BEH130 C18, 3.0 x 100 mm, 3.5µm; column temperature: 45°C; counter ion: dodecyltrimethylammonium)

4 Lipids in dosage nanoparticles

Lipid nanoparticles (e.g. liposomes and composites) are broadly investigated as dosing vehicles for oligonucleotides to improve their stability in dosage formulations as well as their efficiency in reaching their biological targets. In addition to control of dosage accuracy of the active ingredients, it is also important to measure the vehicle lipids in the quality control process of a formulation. Figure 5A shows the schematics of nanoparticles and one of the common types of lipid used in nanoparticle vehicles. The difficulties in the analysis of this type of lipid lie in their usually poor chromatography, and lack of strong chromophore for UV detection.

Proper chromatography of the lipids can often be attained by applying high pH (pH 10 to 12) and alcoholic solvents (ethanol or 2-propanol) in the mobile phase, on a reverse phase column. For detection, the absorption of the amino and ester groups at around 200 nm often provides acceptable sensitivity. Compared with ELSD and RID, UV detection is enormously advantageous in providing the reproducibility and the characteristics for peak identification required in analytical support of non-clinical toxicology research. Figure 5B presents chromatography of a lipid in a dosage nanoparticle as indicated in Figure 5A.

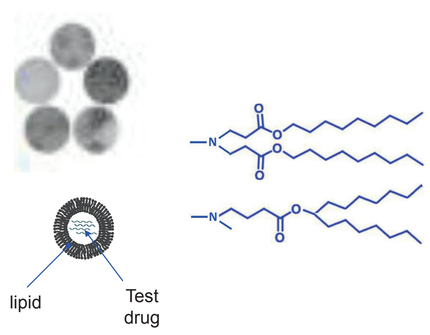

Figure 5A. Schematics of lipid nanoparticles and common type of lipids used in nanoparticle vehicles

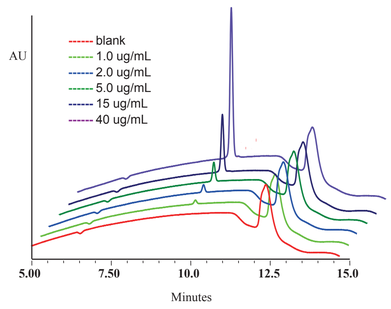

Figure 5B. Chromatography of lipid using UV detection (column: XBridge C18, 3.5 µm, 150x4.6 mm; mobile phase: aceonitrile:isopropanol: ammonium hydroxide, pH 10.5; detection: 204 nm)

5 Acknowledgement

We wish to thank all Technicians in the department of Analytical Chemistry at Charles River Laboratories Montreal ULC (CR MTL), who were involved in the work presented in this poster.