Specific Determination of Oligonucleotide Therapeutics by Dual Ligation Hybridization Assay

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Abstract

Objective: Hybridization assays quantify oligonucleotide-based therapeutics in various biological matrices such as plasma or tissue. Current methods lack to discriminate the parent compound from its modified (PNK) phosphodiester DNA OGNs with the sequence of a published siRNA. All oligonucleotides were DNA and have 5'-G and 3'-O unless otherwise stated. All oligonucleotides were purified by HPLC.

Method: We have developed a dual ligation immunoassay to develop an assay specific for the parent compound.

Results: Full-length, unmodified or phosphorothioate oligonucleotide therapeutics are quantified with the dual ligation hybridization assay. We have demonstrated this mechanism and investigated the specificity of the assay for the analyte. Validation parameters were assessed to document suitability of the method.

Conclusion: We have developed what we believe to be the first hybridization-based assay specific for the parent compound. The methodology is currently being adapted to the determination of oligonucleotide therapeutics by qPCR.

Introduction

Hybridization assays are used to quantify intravenously administered oligonucleotide (ODN) therapeutics at the discovery, preclinical and clinical stages of the drug development process. They include sandwich, competitive, ligation and nucleic acid-based methods. Until now there was no hybridization assay specific for the parent compound from a full-length product (PNK). Instead metabolites short of 1-2 or more nucleotides over the FLP would be detected. Specific hybridization assays are highly desirable as they lead to accurate pharmacokinetic (PK) and toxicokinetic (TK) profiles in support of OGN drug development. Although antisense compounds may be active when truncated of a few nucleotides, the assay should be able to quantify the parent compound of the drug for lead optimization and to determine metabolites in addition to the parent compound. We report the application of a dual ligation-based qPCR method (dual-qPCR) for the quantitation of oligonucleotide analytes on dried blood spots. Two template probes guide the ligating of the analyte onto two generic adapters at either end of the analyte (A). In the dual ligation assay, the parent compound (Template 1) and a metabolite (Template 2) are obtained from a single PCR reaction. The amplified DNA products are used as templates for the individual oligo-probes (Ligation Probe 1 and Ligation Probe 2). The probes are designed to hybridize to the test OGN and two different nucleotides are required for a successful hybridization. Therefore, in order to accurately determine the parent OGN concentration, a sensitive and accurate dual-ligation hybridization assay is needed. The dual-ligation hybridization assay integrates bi-enzymatic ligation of the DNA analyte with a defined set of probes, and constitutes the first parent compound-specific quantitative hybridization assay.

Methods

Bi-enzymatic mechanism of detection

In Figure 3, when the enzymatic reaction is carried out with either individual enzyme, no signal is detected above the lower limit of quantitation (LLOQ). A curve is generated, both PNK and T4 DNA ligase are required. Thus, the signal is generated as schematized in Figure 1, where a bi-enzymatic reaction takes place. Also, the washing conditions satisfactorily eliminate un-ligated OGNs.

Figure 1: Schematic representation of the dual ligation immunoassay

Figure 2: Representative calibration curve with the test oligonucleotide in mouse plasma

Process

The dual ligation-hybridization assay workflow is depicted in Figure 1. The template probe is fully complementary to the test OGN, in addition to having extensions on both the 5' - and 3'-ends that are complementary to the analyte. Ligation Probe 1 is modified for immobilization onto a solid support and hybridized with a phosphorothioate oligonucleotide for fluorescence detection. Ligation Probe 2 is labeled for chemiluminescence signal intensity.

The biological sample containing the test OGN is mixed with the Template Probe and Ligation Probe 1 followed by denaturation/annealing/mixing immobilization onto a 96-well plate. Bi-enzymatic reaction: PNK, ligase and Ligation Probe 2 (ca. 150 ng).

The plates are washed thoroughly to remove un-ligated products at the 5’- and 3’-ends of the Template Probe for in order to signal or count, both signal and quantitatively evaluate the signal using standard reagents.

Results

Standard curve

A representative calibration curve obtained with the dual ligation immunoassay in mouse plasma is shown in Figure 2. The curve spanning range was 0.125 nM (0.4 ng/ml) up to 80 nM (268 ng/ml) using a linear regression model. The behavior and linearity of the curve are comparable to other hybridization assays.

Results in Table 1: Intra-assay precision and accuracy of the analyte in mouse plasma

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<th>% recoveries</th>
<th>Mean (nM)</th>
<th>% RSD</th>
<th>Mean (ng/ml)</th>
<th>% RSD</th>
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</tr>
</tbody>
</table>

Table 1: Intra-assay precision and accuracy of the analyte in mouse plasma

The dual ligation hybridization assay is specific for the parent test OGN and does not significantly detect metabolites. The assay performed comparably in mouse, monkey and human plasma (A, EDTA).

In Table 1, the intra-assay precision and accuracy demonstrate that the method reproduces determinations closely. In Table 2, the inter-assay precision and accuracy demonstrate that the method is reproducible. The precision was assessed for the intra- run using the same OGN in three different lots (Lot #5, Lot #6 and Lot #7).

In Figure 4, the signal generated at different concentrations for the FLP of the analyte versus the same analyte truncated by 1 nucleotide at either the 5'-end or the 3'-end (5’ N-1 or 3’ N-1 metabolites) are compared. For the 5'-end metabolite, the FLP was truncated by 1 nucleotide at the 5'-end and the 3'-end (5’ N-1 metabolite) is detected. For the 3'-end metabolite, the FLP was truncated by 1 nucleotide at the 3'-end and the 5'-end (3’ N-1 metabolite) is detected. In this instance, a low interference of 0.4% was observed for the 5’ N-1 metabolite for the FLP compared to the 3’ N-1 metabolite as seen in Table 2. The dual ligation assay detects the new analyte with no significant interference.

Conclusion

We have developed what we believe to be the first hybridization-based assay specific for the parent compound. The methodology is currently being adapted to the determination of oligonucleotide therapeutics by qPCR.