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Method Development for the Detection of Exicure's AST-008 in Human Plasma

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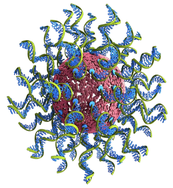
Introduction

AST-008 is a novel spherical nucleic acid (SNA) configuration of a toll-like receptor 9 (TLR9) agonist oligonucleotide, designed to trigger innate and adaptive immune responses that are useful in oncology applications. AST-008 activated key immune cells and cytokines predictive for an anti-tumor effect in a Phase 1 healthy-volunteer study.

A sensitive bioanalytical method was required to determine the concentrations of AST-008 in human plasma with minimal detection of metabolites to support pharmacokinetic analysis in clinical studies.

Figure 1: SNA Structure



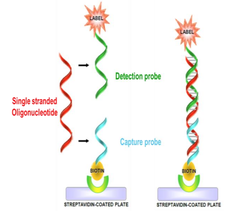
Objective

The concentrations of AST-008 in clinical samples were initially determined by hybridizing a complementary, fluorescently-labeled peptide nucleic acid (PNA) probe and using liquid chromatography with fluorescence detection (LC-FD). All samples, when analyzed with this method, were below the limit of quantification (BLQ) of 10 ng/mL; therefore, a more sensitive method was required. The objective of this study was to develop a bioanalytical method with greater sensitivity, as determined by the lower limit of quantitation (LLOQ). Two hybridization methods (Dual Hybridization and Hybridization-Ligation) and two platforms (Fluorescence and ECL) were compared in order to select the one with greatest sensitivity and selectivity for further validation.

Table 1: Advantages and Limitations of Hybridization Methods

Method	Advantages	Limitations
Hybridization ELISA	No sample clean up (plasma) or minimum sample cleanup (tissue)	Narrower calibration range than chromatographic methods (10- to 50-fold)
	Low reagent costs	
	Very high sensitivity, precision and accuracy	
	Highly selective of parent	Quantitation of parent / total detectable oligonucleotide metabolites (shortmers) not quantifiable in parent assay
	Widely used to support preclinical and clinical studies	
	High target specificity	

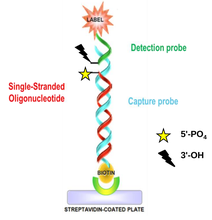
Figure 2: Dual Hybridization ELISA



- Hybridization of oligonucleotide (ON) with capture and detection probes
- Non-Hybridized probe will be washed away
- **Advantage: Enhanced sensitivity and wider dynamic range**
- **Disadvantage: Cross-Reactivity with 3' and 5' metabolites**

Eller, SM et al.(2005) Oligonucleotides,.15 (2) 119–131

Figure 3: Hybridization-Ligation Fluorescence



- Ligation of ON with T4 polynucleotide kinase (PNK) and DNA ligase/ATP
- Non-Ligated probe will be washed away
- **Advantage: Minimal 5'-metabolite cross-reactivity**
- **Disadvantage: Reduced sensitivity**

Yu RZ et al.(2002) Anal. Biochem. 304, 19-25

Results

Table 2: Sensitivity of LC-FD versus Dual Hybridization Methods

	Dual Hybridization	LC-FD
LLOQ (Plasma)	1.000 ng/mL	10.00 ng/mL

- **The Dual Hybridization method was evaluated first using the fluorescence platform and although the LLOQ was lower than the LC-FD method, higher sensitivity was pursued utilizing additional hybridization methods and platforms.**

Table 3: Metabolite Cross-Reactivity with Hybridization-Ligation and Dual Hybridization

		Hybridization-Ligation ECL Method		Dual Hybridization ECL Method	
Nominal Metabolite Concentration (ng/mL)		105.0			
	Run ID	**Metabolite Identity**		**Metabolite Identity**	
		N-1 AST-008	**N-2 AST-008**	**N-1 AST-008**	**N-2 AST-008**
Observed Concentration	**EXI557.33**	7.401	6.573	118.4	104.3
	EXI557.10	7.470	6.493	123.8	95.86
N		2	2	2	2
Mean		7.436	6.533	121.1	100.1
Cross-Reactivity (%)		7.1	6.2	115.3	95.3

$$\%Cross-Reactivity = (Mean\ observed\ concentration) \div (Nominal\ metabolite\ concentration) \times 100$$

- **The hybridization-ligation ECL method had a minimal cross-reactivity with N-1 or N-2 metabolites, whereas the dual hybridization ECL method detected 100% of both metabolites.**

Table 4: Hybridization-Ligation ECL Method Precision and Accuracy

Nominal concentration (ng/mL)	LQCA	LQCB	QC1A	QC1B	QC2	QC3	ULQ
	0.5000	**1.000**	**1.500**	**3.000**	**30.00**	**105.0**	**150.0**
Run ID EXI557.36	0.5110	1.009	1.587	3.186	29.08	111.8	158.4
	0.5066	1.020	1.527	3.174	33.23	113.0	164.3
	0.5293	1.018	1.450	3.039	30.97	114.5	172.4
	0.5343	1.048	1.477	2.926	30.60	115.8	175.4
	0.5519	1.016	1.471	3.075	34.46	125.4	181.0
N	5	5	5	5	5	5	5
Mean	0.5266	1.022	1.502	3.080	31.67	116.1	170.3
SD	0.0183	0.0151	0.0551	0.1065	2.153	5.399	8.979
%CV	3.5	1.5	3.7	3.5	6.8	4.7	5.3
%RE	-5.3	2.2	0.2	2.7	5.6	10.6	13.5

- **The hybridization-ligation ECL method exhibited acceptable precision and accuracy (%CV ≤ 20% and %RE ± 20%) indicating a sensitivity of 0.5000 ng/mL.**

Table 5: Hybridization-Ligation ECL Method Matrix Effect Selectivity

Nominal Concentration (ng/mL)		Blank 0.0000	LQCA 0.5000		LQCB 1.000		QC3 105.0	
Run ID	**Lot #**	AST-008	AST-008	% RE	AST-008	% RE	AST-008	% RE
EXI557.33	**1**	BLQ	0.4553	-8.9	0.8991	-10.1	101.2	-3.6
EXI557.41	**2**	BLQ	0.5232	4.6	1.0490	4.9	114.6	9.2
	3	BLQ	0.3786	-24.3	0.8248	-17.5	93.77	-10.7
	4	BLQ	0.3275	-34.5	0.6893	-31.1	82.11	-21.8
	5	BLQ	0.4091	-18.2	0.8099	-19.0	90.14	-14.2
	6	BLQ	0.4202	-16.0	0.8099	-19.0	96.62	-8.0
	7	BLQ	0.5327	6.5	0.9983	-0.2	116.9	11.3
	8	BLQ	0.4987	-0.3	0.9983	-0.2	115.9	10.4
	9	BLQ	0.4710	-5.8	0.8746	-12.5	102.7	-2.2
	10	BLQ	0.4631	-7.4	0.9237	-7.6	105.1	0.1
	Mean	BLQ	0.4479	-10.4	0.8877	-11.2	101.9	-2.9

- **The hybridization-ligation ECL method exhibited acceptable selectivity (8 of 10 lots with %RE ± 20%) at the 0.5000 ng/mL and 105.0 ng/mL levels.**

Table 6: Hybridization-Ligation ECL Method Dilution Linearity

Nominal concentration (ng/mL)		20000		
Dilution factor		**1:200**	**1:500**	**1:5000**
Concentration after dilution (ng/mL)		**100.0**	**40.00**	**4.000**
Run ID EXI557.38	**Observed concentration (ng/mL)**	22163	24146	21408
		20399	18413	18245
		20175	18966	18305
		20616	19912	18494
N		4	4	4
Mean		20838	20359	19113
SD		901.4	2599	1533
%CV		4.3	12.8	8.0
%RE		4.2	1.8	-4.4

- **Sample dilution was found to be acceptable (%CV ≤ 20% and %Nominal ± 20%) up to 5000 foldusing the hybridization-ligation ECL method.**

Table 7: Validation Summary for the Determination of AST-008 in Human Plasma Using the Hybridization-Ligation ECL Method

Short description of method	Hybridization-Ligation ECL		
Biological matrix	Human Plasma (K_2EDTA)		
Analyte	AST-008		
Calibration concentrations	0.5000 to 150.0 ng/mL		
Sensitivity	0.5000 ng/mL (LOQ QC)		
Lower limit of quantification	LLOQ (ng/mL)	0.5000	
	Between-run accuracy	99%	
	Between-run precision	12%	
	Within-run accuracy	95%	
	Within-run precision	2%	
Between-run accuracy	91% to 99%		
Between-run precision	4% to 12%		
Within-run accuracy	90% to 95%		
Within-run precision	1% to 5%		
Matrix effect	No significant interference was observed in 9 out of 10 individual human plasma lots: acceptance criteria were met at all tested levels (Blank [un-spiked], LOQ QC and QC3). Acceptance criteria were met at all tested levels (Blank [un-spiked], LOQ QC and QC3) in hemolyzed (up to 2%) human plasma and lipemic (>300 mg/dL triglycerides) human plasma.		
Metabolite cross-reactivity	Minimal cross-reactivity observed for N-1_AST-008 (4%) and N-2_AST-008 (1%) at QC3 level (105.0 ng/mL).		
Metabolite interference	No effect on the determination of AST-008 in human plasma tested at QC1 and QC3 levels in the presence of either metabolite N-1_AST-008 or N-2_AST-008.		
Hook effect	No hook effect observed up to 20000 ng/mL.		
Dilution linearity	DQC1 at 20000 ng/mL was used for dilution factors of 1/200 and 1/5000, whereas DQC2 at 200.0 ng/mL was used for dilution factor of 1/5.		
		%Nominal	%CV
	Diluted 5-fold	86%	3%
	Diluted 200-fold	87%	4%
	Diluted 5000-fold	95%	3%
Whole blood stability	Reported up to 2 hours on ice/water bath.		
Stock stability at -20 °C Nominal	Reported up to 19 days at -20 °C nominal. Extended period will be conducted on a later date.		
Short-term matrix stability at 22 ºC Nominal	Reported up to 26.2 hours at ambient room temperature.		
Freeze-thaw matric stability	Reported up to 5 cycles.		
Long-term matrix stability at -20 °C Nominal	Reported up to 11 days. Extended period will be conducted on a later date.		

- **The hybridization-ligation ECL method is fully validated with respect to accuracy, precision, selectivity (matrix effects, metabolite cross-reactivity and interference), hook effect, dilution linearity and stability.**

Conclusions

- ✓ The hybridization-ligation ECL method to measure AST-008 concentrations in human plasma was successfully validated with the range of 0.5000 ng/mL to 150.0 ng/mL.
- ✓ This method is 20-fold more sensitive than the PNA probe, LC-FD method that was formerly validated for the same analyte.
- ✓ The level of sensitivity obtained using the hybridization-ligation ECL method enabled the detection of low concentrations of AST-008 in clinical study samples that had previously been undetectable using the PNA probe LC-FD method.

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