

Validation of Bioanalytical Capillary Gel Electrophoresis Methods for the Determination of Oligonucleotides

Guest Authors

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To quantitate antisense oligonucleotides in biological matrices by capillary gel electrophoresis, Good Laboratory Practices-compliant laboratories must validate the method of analysis according to guidelines set by the U.S. Food and Drug Administration. The guest authors describe how this validation is performed routinely in their laboratory.

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Antisense oligonucleotides have emerged as a promising class of drugs for treating diseases ranging from viral infections to different kinds of cancer (1). In antisense therapy, an oligonucleotide — in short, oligo — is synthesized with a sequence that complements a section of a target deoxyribonucleic acid (DNA) or messenger ribonucleic acid (mRNA) and prevents their expression (2). As antisense therapy matures, scientists need a robust analytical technique to determine oligos and their metabolites in various matrices. Capillary gel electrophoresis has emerged as the method of choice for determining oligos in many biological matrices (3,4).

The analytical methods used during drug development must be validated according to guidelines set by the various regulatory agencies involved in the approval process for antisense drugs. In this “Validation Viewpoint” column, we will look at the regulatory requirements, with a special emphasis on those of the U.S. Food and Drug Administration (FDA), necessary to validate capillary gel electrophoresis methods designed to determine oligonucleotides in biological matrices.

Validation of Bioanalytical Methods

In May 2001, FDA issued a guidance document about validating bioanalytical methods, a document intended to assist scientists involved in submission of investigational new drug applications (INDs) and new drug applications (NDAs), among others (5). The latest version of this document, titled *Guidance for Industry, Bioanalytical Method Validation*, represents, by FDA’s own words, “the Food and Drug Administration’s current thinking on this topic” and

has been adopted widely in the pharmaceutical and related industries.

According to FDA, “Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix . . . is reliable and reproducible for its intended use” (5).

The validation of a bioanalytical method should include the following parameters: calibration standard curve, intra- and inter-batch precision and accuracy, recovery, selectivity, carryover, lower limit of quantitation, and stability of the analyte under relevant conditions (Table I).

The calibration standard curve should be assessed by analyzing at least six to eight standards in addition to a blank (matrix processed without the internal standard) and a zero standard (matrix processed with the internal standard), all of which should be prepared in the same biological matrix as the samples under study. The simplest regression line should be used. Acceptable correlation coefficients should be greater than 0.95, and back-calculated concentrations of the standards should be within $\pm 15\%$ of their theoretical values ($\pm 20\%$ for the lower limit of quantitation standard).

The precision and accuracy can be determined by analyzing a minimum of five spiked samples at each of three concentrations that correspond to the low, middle, and high ends of the calibration curve. For intra- and inter-batch precision and accuracy, these two parameters are assessed within a single run and between several runs, respectively. The acceptance criteria are $\pm 15\%$ for accuracy (expressed as percent relative error) and 15% or less for precision (coefficient of variation).

Recovery is a measure of the response of an analyte that has been extracted from a biological matrix relative to the response of a standard solution of the same concentration. Recovery should be assessed at low, middle, and high concentrations. Scientists commonly report recovery results without setting acceptance criteria. The consensus is that a high recovery is not crucial as long as the precision and accuracy are acceptable.

For an assessment of selectivity, a minimum of six different lots of blank matrix should be analyzed. Interference in each of the blank samples should be compared with the lower limit of quantitation standard. To be considered acceptable, any interference must be 20% or less of the lower limit of quantitation standard.

The lower limit of quantitation can be established by analyzing at least five replicates of the lowest concentration standard in the calibration curve. Users need to calculate the precision and accuracy and compare the responses of the lower limit of quantitation standard with those of the blank matrix.

Stability of analytes under the conditions for the actual study sample should be assessed. This parameter includes stability in the biological matrix under the study's storage conditions, effect of at least three freeze and thaw cycles, stability in injection medium, in-process stability, and stability of the stock solutions used during analysis. Stability can be assessed by analyzing quality control samples at low and high concentrations, initially and then after storage for a predefined period of time. An acceptable difference between the stored and fresh samples should be within $\pm 15\%$.

Applying the FDA Guidance Document to Capillary Gel Electrophoresis

According to the FDA Guidance Document, "The recommendations [provided in the document] can be adjusted or modified depending on the specific type of analytical method used" (5). In the case of capillary gel electrophoresis, analysts can apply most recommendations in a fashion similar to those of conventional chromatographic techniques with minor differences, including slightly different acceptance criteria.

Normalized peak area commonly is used in capillary gel electrophoresis as the analytical signal. The normalized peak area is defined as the corrected peak area of an analyte divided by the corrected peak area of the internal standard. The corrected peak area refers to the peak area response divided by the migration time.

In the following sections, we describe how we apply the FDA Guidance Document in our laboratory (6) to validate bio-analytical capillary gel electrophoresis methods in compliance with Good Laboratory Practices (GLPs) (*21 CFR Part 58*) (7). We discuss each validation parameter and highlight the differences between conventional chromatographic techniques, when applicable.

Calibration curve: We use two calibration curves. Each curve contains at least six to eight non-zero standards because of the nonlinearity of the response throughout the concentration range. We perform linear regression analysis on normalized peak area versus theoretical concentrations. Linearity is considered acceptable if the correlation coefficient (r^2) is 0.95 or greater and if the back-calculated concentrations of the stan-

Table 1: Summary of validation parameters

Validation Parameter	Acceptance Criteria
Calibration curve	$r \geq 0.98$ and relative error within $\pm 20\%$
Intra-batch precision and accuracy	$n = 6$, one batch, three concentration levels, relative error within $\pm 20\%$, and coefficient of variation $\leq 20\%$
Inter-batch precision and accuracy	$n = 18$, three batches, three concentration levels, relative error within $\pm 20\%$, and coefficient of variation $\leq 20\%$
Selectivity	Five of six lots of blank matrix, any interference $\leq 25\%$ of lower limit of quantitation standard
Carryover	Carryover $\leq 25\%$ of lower limit of quantitation standard
Lower limit of quantitation	Six standards, relative error within $\pm 25\%$, coefficient of variation $\leq 25\%$
Stability	Stored samples results within $\pm 20\%$ of fresh results

dards are within $\pm 20\%$ of their theoretical values.

Precision and accuracy: We analyze three sets of quality control samples for low, middle, and high concentrations with each of the two curves. We repeat this process on three occasions to determine inter-batch precision and accuracy. For accuracy, the back-calculated concentrations of five of the six quality control samples at each level must be within $\pm 20\%$ of their theoretical values. For precision, the coefficient of variation (CV) for all the quality control samples at each level must be 20% or less. This requirement is somewhat different from conventional chromatographic methods, in which most laboratories tend to use a 15% criterion. Table II shows typical precision and accuracy results.

Selectivity: We assess selectivity by analyzing six lots of blank matrix. Any signal at the migration time of the analyte in five of the six lots must be $\leq 25\%$ of the lower limit of quantitation standard. This value can be compared with a 20% criterion used for most conventional chromatographic methods.

Carryover: We assess carryover by injecting a blank solution immediately after an injection of a high concentration standard. Any signal at the migration time of the oligonucleotide analyte must be 25% or less of the lower limit of quantitation standard.

Lower limit of quantitation: We establish the lower limit of quantitation by analyzing six standards at the lowest concentration of the calibration curve. For the lower limit of quantitation to be acceptable, the back-calculated concentrations of five of the six standards must be within $\pm 25\%$ of their theoretical values. For precision, the coefficient of variation must be 25% or less. We also require a signal-to-noise ratio greater than 3.

Stability: We assess the stability in the bioanalysis matrix by analyzing quality control samples at low and high concentrations, initially and then after storage at conditions that correspond to the study samples. We consider the stability to be acceptable if the difference between the stored and fresh samples is within $\pm 20\%$.

In-process stability is an important parameter for oligos. Sample pretreatment can include more than one solid-phase extraction step and overnight evaporation followed by reconstitution and desalting. Because of practical considerations, the samples could spend time on the bench

between these pretreatment steps. Thus, it is necessary to prove the integrity of the analytes after each step of the process. The difference between stored and uninterrupted processed samples should be within $\pm 20\%$.

We assess the injection medium stability by analyzing processed samples when they are fresh and then after being stored under study conditions. The difference between the stored and fresh samples must be within $\pm 20\%$ to be acceptable.

Partial Validation

Partial validation, as opposed to a full validation, can be performed when the analytical method has been validated for an analyte in one biological matrix and if one or more of the following applies:

- We are determining the same analyte in the same matrix but for a different species; for example, rat plasma versus dog plasma.
- We are determining the same analyte in the same species but for a different matrix; for example, dog plasma versus dog urine.

- We are determining the same analyte in the same matrix and species but using a different anticoagulant; for example, sodium EDTA versus sodium citrate.
- We are using a rare matrix; for example, bone marrow.
- We are making minor changes to the analytical method.

The extent of work necessary for a partial validation should be judged on a case-by-case basis. Typically, a partial validation would include assessing all the parameters included in a full validation, as described above, with the exception of inter-batch precision and accuracy and stability, except for cases in which stability could be affected.

Chain-Shortened Metabolites

In the body, antisense oligos are broken down into their chain-shortened metabolites, which individually are called ($n - x$)-mer metabolites (where x is the number of nucleotides that have been removed from the parent compound), usually through catabolic pathways (Figure 1) (8). Researchers have shown that several of the

Table II: Typical results for intra- and inter-batch precision and accuracy*

Nominal Concentration (μM)	Relative Error (%)			Global Mean Relative Error (%)	Global CV (%)
	Batch 1	Batch 2	Batch 3		
1.50	1.6	-15.0	3.9		
1.50	-5.8	-7.1	8.3		
1.50	-6.7	-6.4	8.2		
1.50	-4.6	-6.3	4.1		
1.50	-6.3	-5.2	6.0		
1.50	-3.8	-1.6	6.2		
Mean relative error (%)	-4.3	-6.9	6.1	-1.7	
CV (%)	3.2	4.7	1.8		6.6
5.00	4.8	-5.9	4.1		
5.00	5.9	-10.2	0.8		
5.00	-13.6	-14.1	1.9		
5.00	-5.4	-9.2	7.2		
5.00	-3.0	-8.3	6.9		
5.00	-14.0	3.3	0.5		
Mean relative error (%)	-4.2	-7.4	3.6	-2.7	
CV (%)	9.0	6.4	2.9		8.3
9.00	-7.4	1.6	-3.6		
9.00	-6.9	-4.3	-2.2		
9.00	-9.3	1.1	-4.7		
9.00	-7.7	-3.4	-2.4		
9.00	-9.1	-10.3	-0.6		
9.00	-6.5	0.1	-2.1		
Mean relative error (%)	-7.8	-2.5	-2.6	-4.3	
CV (%)	1.2	4.6	1.5		3.1
r^2 of calibration curve	0.999	0.999	1.000		

*The values shown are for a parent 20-mer oligo from a capillary gel electrophoresis analysis method from human plasma. The method subsequently was used to analyze samples from clinical trials for a submission to FDA.

high-order, chain-shortened metabolites retain their antisense activity (9). Thus, in a toxicokinetic or pharmacokinetic study, scientists must be able to quantify reliably at least the three or four highest order metabolites. Analysts must take this requirement into consideration whenever they develop and validate an analytical method for the determination of oligos. In addition to clearly distinguishing different metabolites, a method should be capable of being validated with respect to quantitating the individual metabolites.

In our laboratory, we have taken two approaches to reliably quantitate the individual chain-shortened metabolites. In our opinion, both approaches are acceptable for validating analytical methods. In the first approach, we prepare calibration curves using standards of each of the metabolites and the calibration standards of the parent oligo. The metabolites in the study samples then are read from their respective calibration curves. In the second approach, we prepare a calibration curve only for the parent oligo; the metabolites in the study samples then are read from the parent calibration curve. We apply a correction factor to account for the difference in absorptivities between the parent and the metabolites.

Capillary Gel Electrophoresis Instrument Validation

Capillary gel electrophoresis instruments depend upon microprocessor technology and software to ensure proper instrument control, data acquisition, and data process-

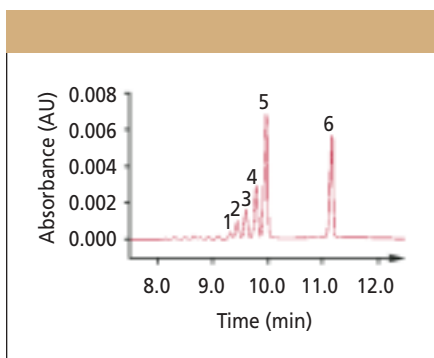


Figure 1: Electropherogram showing a 20-mer oligonucleotide and its chain-shortened metabolites in human plasma following IV infusion. Capillary gel electrophoresis conditions: capillary: LPA coated, 31 cm \times 75 μ m, 20 cm effective length; gel type: ssDNA 100-R; running buffer: Tris borate, 7 M urea; injection: electrokinetic, 10 kV; separation: 20 kV; detection: 260 nm. Peaks: 1 = 16-mer, 2 = 17-mer, 3 = 18-mer, 4 = 19-mer, 5 = 20-mer, 6 = internal standard.

ing. FDA requires that these systems be validated before use on studies geared to regulatory submissions. It is beyond the scope of this "Validation Viewpoint" column to discuss this undertaking; however, we will mention our laboratory's key requirements for validation. These requirements are based on FDA's *Validation Draft Guidance for Industry 21 CFR Part 11* (10).

Instrument validation has four important steps:

List the system requirements: The specific needs for the intended use of the instrument should be predefined and documented. The focus should be on ensuring record authenticity, integrity, and confidentiality.

Properly document all validation activities: This step should include a master validation plan, a procedure that lists the detailed steps of the validation, a validation report, and a description of the validation approval process.

Test the system: This step is to ensure proper installation and functionality under normal and stress conditions. The data integrity, audit trail, security, and archiving also should be checked. The testing should follow the procedures mentioned in the documentation step.

Revalidate: Assess any changes in the system after initial testing for a possible revalidation.

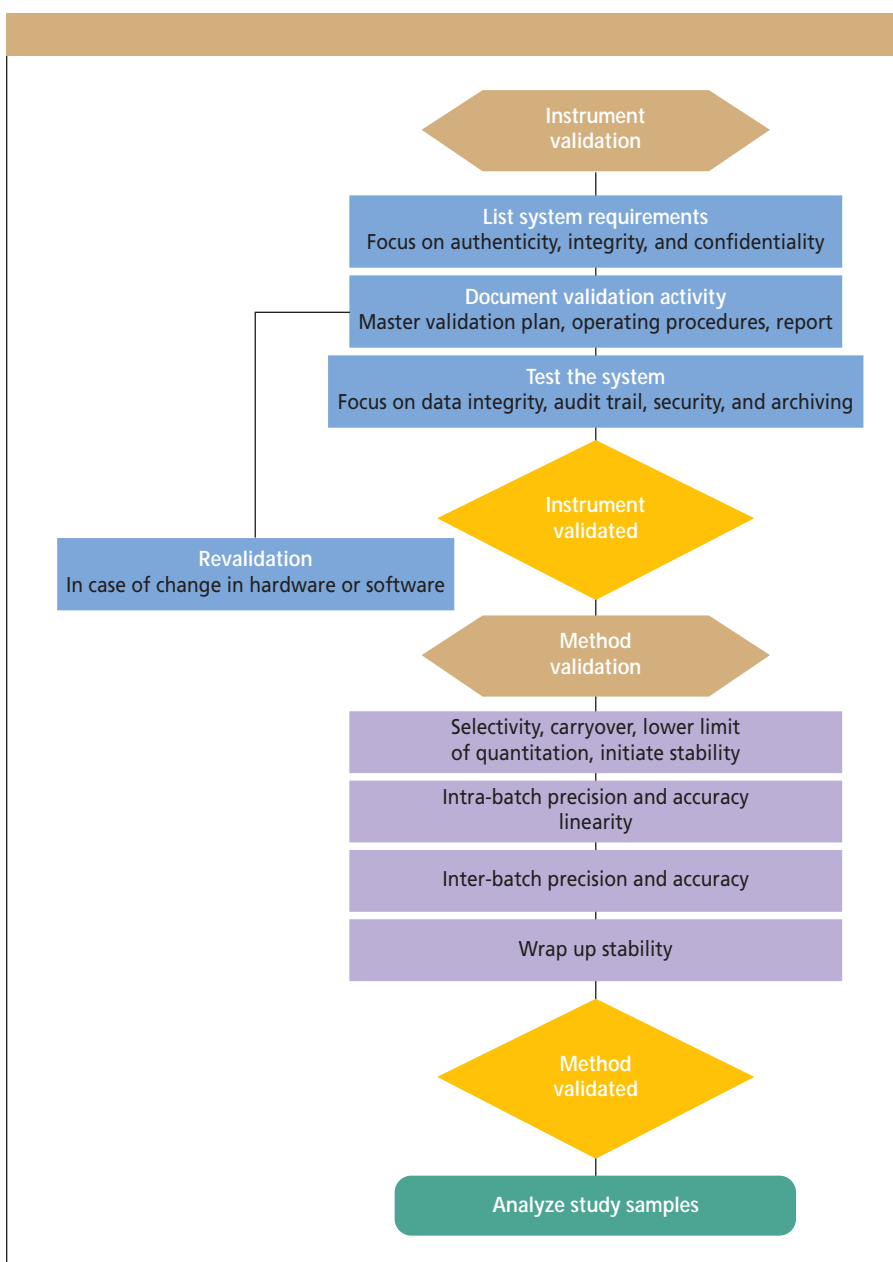


Figure 2: Validation flow chart.

Conclusion

To analyze oligos and their metabolites in biological matrices by capillary gel electrophoresis in a GLP environment, scientists first must validate an analytical method and the capillary gel electrophoresis instrument itself (Figure 2) in accordance with FDA guidelines. To be meaningful, any oligo method validation must take the quantitation of the chain-shortened metabolites into consideration.

Please note that the content of this column is the opinion of the guest authors. It represents their interpretation of the FDA Guidance Document and by no means necessarily reflects the views of the U.S. Food and Drug Administration.

References

- (1) D.D. Ma, T. Rede, N.A. Naqvi, and P.D. Cook, *Biotechnol. Ann. Rev.* **5**, 155–196 (2000).
- (2) D.W. Green, H. Roh, J. Pippin, and J.A. Drebin, *J. Am. Coll. Surg.* **191**(1), 93–105 (2000).
- (3) S.H. Chen and J.M. Gallo, *Electrophoresis* **19**(16-17), 2861–2869 (1998).
- (4) R.S. Geary, J. Matson, and A.A. Levin, *Anal. Biochem.* **274**, 241–248 (1999).
- (5) *Guidance For Industry, Bioanalytical Method Validation* (U.S. Department of Health and

Human Services, U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Rockville, Maryland, May 2001).

- (6) P. Nader, G. Kremmydiotis, J. Tremblay, G. Lévesque, and A. Bartlett, "Validation of a Capillary Gel Electrophoresis Method for the Determination of an Oligonucleotide and Its Metabolites in Human Plasma," abstract presented at CE in the Biotechnology and Pharmaceutical Industries: Practical Applications for the Analysis of Proteins, Nucleotides and Small Molecules, Boston, Massachusetts, 19–21 August 2001.
- (7) *21 Code of Federal Regulations, Part 58* (U.S. Food and Drug Administration, U.S. Government Printing Office, Washington, D.C., 1 April 2001).
- (8) M. Gilar, A. Belenky, D.L. Smisek, A. Bourque, and A.S. Cohen, *Nucleic Acids Res.* **18**, 3615–3620 (1997).
- (9) S.H. Chen, M. Qian, J.M. Brennan, and J.M. Gallo, *J. Chromatogr. B* **692**, 43–51 (1997).
- (10) *21 Code of Federal Regulations, Part 11* (U.S. Food and Drug Administration, U.S. Government Printing Office, Washington, D.C., March 2000).

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The columnists regret that time constraints prevent them from responding to individual reader queries. However, readers are welcome to submit specific questions and problems, which the columnists may address in future columns. Direct correspondence about this column to "Validation Viewpoint," LCGC, 859 Willamette Street, Eugene, OR 97401, e-mail lcgcedit@lccmag.com.