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Val-CiD Best Practice Guide: CD spectroscopy for the quality control of biopharmaceuticals

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1. Introduction

This report is a result of part of the work done under the project titled "Development and Validation of Circular Dichroism Methods for the Quality Control of Biopharmaceuticals" (also known as "Val-CiD") which was formulated in response to the Invitation to Tender (ITT) GBBK/C/019/00009, as part of the DTI's first "Measurements for Biotechnology" programme. The project was led by the National Institute for Biological Standards and Control and delivered by a broad consortium of interested parties including the National Physical Laboratory, GlaxoSmithKline, Imperial College, University of Warwick, Chiralabs, Elliot Scientific and Jasco. A number of linked documents have been produced as a result of this project, and they are listed in Table 1.

This report provides guidance on the problems that may arise in the use of circular dichroism in a biopharmaceutical QC environment, and makes recommendations as to how to avoid or minimise these problems. Further details, or experimental support, are provided in the accompanying appendices.

Document	Title
Best Practice Guide	Val-CiD Best Practice Guide: CD spectroscopy for the quality control of biopharmaceuticals
Appendix A	CD spectroscopy: an inter-laboratory study
Appendix B	The Use of Chemical Calibrants in Circular Dichroism Spectrometers
Appendix C	Data analysis approaches for CD Spectroscopy

Table 1 Documents produced under this study.

Reports may be obtained directly from NPL, or from the NPL[1] or MfB[2] websites.

1.1. The well characterised biological product

In the past, manufacturing of biological products (vaccines and biotherapeutics) was dependent on rigorously maintaining the manufacturing process used to produce material used in the clinical trial – the "Gold Standard" assessment of safety and clinical efficacy. The mantra was "the product is the process". This was combined with a biological assay of the final product, usually *in vivo*, to assess the consistency of the batch of product with respect to potency or safety in an appropriate animal model. On this basis, changing a step in the manufacturing process, even a column used in purification, resulted in a "new product" for which some kind of clinical trial or bridging study was required. Thus bringing antiquated production processes up to date was an expensive task to be avoided if at all possible. An underlying assumption in this paradigm, true at the time, was that methods to assess the purity, structure and status of the final product were insufficiently powerful to provide enough data to ensure comparability of, for example, successive batches of a product, or comparability of batches produced using slightly different manufacturing processes.

[1] http://www.npl.co.uk/biotech

[2] http://www.mfbprog.org.uk/

The concept of the well characterised (or well-specified) biological product developed in parallel with the introduction of products manufactured using recombinant technology and the availability of increasingly powerful analytical methods, as it became possible to assess the consistency of manufacture of successive batches by *in vitro* or physicochemical methods alone.

Clearly the extent to which this is true depends on the complexity of the product being assessed and it has been most widely applied to "simple" biotherapeutic products such as recombinant insulin or human growth hormone, or polysaccharides used in vaccine manufacture.

At a general level it is possible to define the types of information that are required to assess the consistency of manufacture of a product (a recombinant glycoprotein, for example) and the methodology that can be used to determine this.

Information required	Appropriate technologies
Is the protein sequence correct?	• Accurate mass of intact protein by MALDI mass spectrometry • Peptide mapping, perhaps identifying peptides by mass spectrometry.
Are the post-translational modifications (PTMs) consistent between batches?	• Glycan mapping using HPAEC or fluorophore labelling techniques. • Mass spectrometry of intact proteins or proteolytic digests • Lectin array methods
Does the product contain any impurities?	
• Product related impurities	• Peptide mapping approaches
• Process related impurities	• Immunological methods
• Host-cell related impurities	• Immunological and PCR methods
Are the secondary and tertiary structures correct?	• Circular dichroism spectroscopy • FT-IR or Raman spectroscopy • NMR • Binding of specific mAbs, ligands or receptors.
Does the product contain any aggregates?	• Size exclusion chromatography, with UV or MALLS detection • Analytical ultracentrifugation.
Does the product retain its correct functional activity?	
• Enzymatic activity	• Enzyme assay
• Receptor binding activity	• Physicochemical or *in vitro* cell binding assay
• Ligand-binding activity	• Fluorescence binding assay, SPR

Table 2 Information required in biopharmaceutical product QC.

1.1. The role of circular dichroism

Of the required information and the techniques listed in the Table above, those used to assess secondary and tertiary structure are the least well developed for use in a regulatory environment. All of the physical techniques, circular dichroism, FT-IR and Raman methods

are simplified and incomplete representations of extremely complex data. Whilst CD spectra are probably the simplest to obtain, they are the super-position of multiple broad peaks for which there is no simple correlation with any given structural factor. They have relatively poor signal-to-noise and instrument calibration has not been fully regularised.

To be appropriate for the task in hand – assessing the consistency of batches of a biopharmaceutical product – it is not necessary to have a full understanding of the relationship between the structure and the spectrum generated. It *is* necessary to have objective means to distinguish whether two spectra are essentially the same (the differences are below statistical significance) or are probably different. To achieve this it is necessary to know the sources and magnitudes of the errors that arise – including both random and systematic errors. It is also important to understand how factors external to the spectroscopic measurement can affect the result. Understanding the origins of the errors can allow procedures to be put in place to minimise errors, improve the quality of the data and increase the "sensitivity" of the technique – the minimum spectral variation that is recognised as significantly different. On a product specific basis, it is necessary to know whether likely structural changes (i.e. those structural changes which may result from realistically occurring problems) result in observable spectral differences, or whether observed spectral differences correlate with variations in the clinical efficacy or safety of the product. These product-specific factors are beyond the scope of this project.

However this does lead to another possibility. The definition of "essentially identical" suggests a "yes-or-no" result. This would be true, for example, if the correlation coefficient between two spectra were calculated and was required to be above a certain threshold specification for a pass. Practically, however, mild thermal abuse of a protein biotherapeutic might lead to different structural change than brief treatment at an inappropriate pH. Only one of these treatments might compromise the clinical efficacy of a product, and their circular dichroism spectra could be different, although both might fail a "yes/no" test based on a single specification. The alternative is to use a more sophisticated analysis of the circular dichroism data, such as a cluster analysis. Different clusters may arise from different mistreatments or process failures, and whilst some "clusters" may be clinically effective, others may not (see Appendix C for examples).

Clearly the development of methodology to objectively identify structural changes can also be applied to monitor product stability during development or, for example, product consistency after process changes. The technology and guidelines developed in this project should also be appropriate for other spectroscopic tools such as FT-IR or, for example, size exclusion chromatography to analyse micro-heterogeneous products.

1.2. Instrument calibration

There are parameters in a CD instrument requiring calibration – ellipticity and wavelength. Wavelength errors can be linear, with the same error being present at all wavelengths measured, or non-linear with the magnitude of the error being wavelength dependent. In the latter case, calibration needs to be carried out at multiple wavelengths and an appropriate correction curve created. As this is likely to depend upon the monochromator, the shape of the correction curve can be expected to be instrument specific. Traditionally, calibration of ellipticity has been performed at a single wavelength, and the absence of wavelength dependent errors assumed. Increasingly, there is a demand for more sophisticated calibration approaches, and these issues are addressed in Appendices to this report.

1.3. The framework within which this project developed

As it is clearly impossible to cover all eventualities, this project was framed in the context of a possible practical application. This was of a biopharmaceutical manufacturer, operating a single CD instrument, producing a product for which circular dichroism provides a realistic means to assess the consistency of the secondary structure of a protein or glycoprotein product. The product is available in non-limiting quantities and is present in a buffer that does not significantly complicate spectral measurements. Instrument calibration is by conventional means.

For a pharmacopoeial method, requirements would be more complex as the methodology and specifications must be transferable between different laboratories using different instruments, between operators and reagents. This has much more complex implications for instrument calibration and operation. As a preliminary to such studies, we carried out an inter-laboratory study to assess the differences in the spectra obtained using different instruments but with a range of common samples (see Appendix A).

1.4. Literature information on methods to optimise data collection

The quality of the original circular dichroism data is fundamental to the analysis. Poor quality data will have high variability and noise, and reduces the ability of the data analysis package to distinguish small but real spectral differences – i.e. the overall analysis method loses sensitivity to small structural differences. On the other hand, any routine analytical method must balance the quality of the data against the time and effort required to obtain it. There are a number of published sources that provide guidance on protocols and instrument settings to optimise data collection. These are listed below: -

Books and chapters in books

Rodger, A., and Ismail, M. (2000). Introduction to circular dichroism, in: "Spectrophotometry and Spectrofluorimetry" (Gore, M. G, Ed.), pp.99-139, Oxford University Press, Oxford, UK

'Circular dichroism' chapter by Stephen Martin (NIMR) in "Proteins: Labfax", N.C. Price (Ed.), Academic Press, Oxford, 1996.

'Circular dichroism' chapter by Alex Drake in "Protein-Ligand Interactions: Structure and spectroscopy - a practical approach", (Harding, S. E., Chowdhry, B., Eds.), Oxford University Press. ISBN 0199637474

Burgess C., Frost T., "Standards and Best Practice in Absorption Spectrometry", Blackwell Science 1999.

PART VII: MOLECULAR ABSORPTION SPECTROSCOPY, ULTRAVIOLET AND VISIBLE (UV/VIS). This IUPAC document contains sections (nos. 5 & 6) discussing 'factors influencing precision of absorbance measurements' and 'factors influencing accuracy of absorbance measurements', and much of this is relevant to CD measurements too (see also section 7 / table X.1 in

there). http://www.iupac.org/reports/V/spectro/partVII.pdf

Information provided by instrument manufacturers

Useful information including technical reports may be found on the web sites of CD instrument manufacturers.

Jasco Europe: http://www.jasco-europe.com/

Olis: http://www.olisweb.com/

Applied Photophysics: http://www.photophysics.com/

1.5. The Good Practice Guide

Through an understanding of the theory the operation of the instrument being used and by optimal choice of protocols and conditions, the quality of the data produced by CD experiments can be improved. Optimising the quality of the data to obtain minimal noise and maximal resolution in the minimal time produces the best data for objective comparisons. If the data sets are sufficiently poor, no two spectra can be shown to be significantly different. Careful and reproducible calibration of the instrument also improves the comparability of data and the ability to compare data collected on different instruments.

Similarly, careful choices of methodologies to pre-process the raw spectral data and to carry out an analysis to objectively determine the whether two spectra or series of spectra are statistically different. The Guide to Good Practice is an attempt to indicate how this should be carried out. The recommendations are detailed in section 3.

1.6. Recommendations for further work

- To make available reference data sets of near and far UV CD spectra from samples created to have small spectral differences consistent with those that might occur in biopharmaceutical production. The intended use of such data sets will be to allow scientists implementing novel data pre-processing or data comparison packages to assess the ability of that package to distinguish, in a statistically significant manner, whether they are different or not. This will provide a means to validate the pre-processing and data analysis packages, and assess their ability to distinguish small differences, without complications from differences in data collection approaches.
- To make available (or to define how to prepare) samples with small spectral differences which can be analysed using the combination of data collection, data pre-processing and data analysis, to assess and validate the complete package. Such samples might be proteins at slightly different concentrations, or samples of one protein "spiked" with small amounts of another to slightly change the CD spectra.
- More work is required to explore different data analysis and statistical packages, and specifically to assess their ability to reduce the subjective observation of two clusters (derived from principal component analysis approaches) to a single number, indicating the likelihood that the spectra of the test sample and the reference sample(s) are different.
- There is a need to extend the work comparing different instrument facilities to an international arena, and push for the acceptance of the conclusions on best practice to an international audience.
- More work is required to develop a consensus within European and international regulatory authorities on the use of CD to assess the secondary structure of recombinant products, based in the knowledge that methodology is available to objectively compare the spectra and indicate which samples/batches are significantly different from others (and the batches used in clinical trials particularly).

2. Criteria for deciding whether the methodology is "fit-for-purpose"

2.1. Introduction

The growing use of physicochemical methods to characterise and control the quality of biopharmaceutical products requires that the individual methods be fully validated to levels acceptable to the regulatory authorities worldwide. The validation package must include the suitability of the instrument to perform its work, its calibration, specifications on the quality requirements for acceptable data, and the data packages used to analyse the data.

Circular dichroism (CD) is an optical technique which quickly, simply and with high sensitivity provides information on the secondary and tertiary structures of protein and glycoprotein products, and has a role in stability and formulation studies. The spectra tend to have relatively poor signal-to-noise and arise from a series of overlapping broad peaks. Although widely used in research and characterisation studies of proteins, glycoproteins, nucleic acids and small molecules, and in assessing stability and interactions, there has been little work to develop objective means to discriminate between spectra. Thus CD has not been widely used for quality control purposes.

For circular dichroism to be used as a quality control tool, it is necessary to reduce a complex data set to a single number which can be compared with a specification which reflects the probability that the spectrum of the text sample and either a historical reference spectrum, or the spectrum of a reference sample obtained concurrently, are different. This in turn requires an understanding of the spectral variability arising from spectral noise, instrument drift and day-to-day variability, and the variability in the spectra of batches of samples that are clinically acceptable. In the production of this document, we have assumed that all the spectra are generated using a single instrument, and have ignored instrument-to-instrument variability.

This document is intended to help in the validation of this process.

If the spectra of test and reference samples are shown to be significantly different, this might be a trigger for rejection of the batch, or for further investigations. Whether the spectral differences observed are sufficient to cause unacceptable changes in functional properties needs to be decided on a product-specific basis, and is beyond the scope of this document. We also recognise that unacceptable changes in functional properties may occur without significant changes in the CD spectra, in which case other assays to assess functional properties will be required. It may also be desirable to gain information that defines in what way the spectra are different (e.g. to distinguish between thermally- or chemically-induced changes in protein structure), or to quantify these differences.

2.2. Approach

The data collection and analysis process can be broken down into four basic steps.

1. Collection of the data from the test sample for comparison with historical reference data or the spectrum of a reference samples run concurrently. It is necessary to know the spectral variability that arises from instrument noise, cell artefacts and other sources (see section 3).

2. Data pre-processing. This may include baseline subtraction, normalisation of the intensity for calibration errors or differences in cell path length, or adjustments to the wavelength axis to account for calibration or other instrument errors.

3. Reduction in the dimensionality of the data so as to highlight those factors or dimensions that are most variable. Appropriate methodologies might include principal component analysis or deconvolution of the spectrum to Gaussian peaks in wavenumber space, or elements of secondary structure determined by appropriate software.

4. Statistical analysis of the reduced dimensionality data to provide an objective statistical estimate of the probability that the spectra (as a surrogate for the samples) are different, and which can serve for comparison with the agreed specification. This process might also include an estimate that of the probability that the sample belongs to a particular sub-group, that might arise from a specific factor in the production process.

In addition, there are several peripheral processes, which need to be validated

- Instrument validation and calibration – is the instrument capable of obtaining data of a sufficiently high quality?

- Sample preparation – can samples be prepared with appropriately low inherent variability that meaningful data can be obtained?

- Reference data set – production of a reference or training data set, which contains spectra from samples known to be of good quality, and reflects typical experimental variability. It may also contain samples known to be of poor quality to assist in the identification of manufacturing problems.

We propose a three-phase approach to assessing the fitness-for-purpose of the experimental and data analysis processes:-

1. To make available near- and far-UV circular dichroism reference data sets that will allow the data analysis package to be validated. These data sets will contain CD spectra that differ slightly one from another to an extent which has been shown to be distinguishable by well-described software approaches, and which should be distinguishable, at a defined statistical level, by the user.

2. Similar near and far-UV circular dichroism reference data sets which will be made available to a variety of pre-processing approaches to be assessed, and which should, after appropriate data analysis steps be distinguishable or not, depending on the combination of spectra compared.

3. To recommend experimental tests to generate data sets in-house to be analysed by data analysis packages and data pre-processing packages validated in points 1 and 2 above. A validated process should be capable of collecting and analysing the data to distinguish the small differences generated by the test methods. This aspect should validate the ability of the instrumentation and data collection protocol to acquire spectra of sufficient quality that defined small structural differences can be distinguished by the combination of spectroscopy, pre-processing and data analysis. In the future, reference standards may be produced.

As emphasised previously, this document can only deal with the generic problem of the ability to distinguish whether two spectra are significantly different. Whether this difference,

or what magnitude or type of difference, is clinically significant will be product-specific and needs to be assessed by individual product manufacturers.

Availability of reference data sets

These near- and far-UV CD reference data sets are currently being developed as part of the M*f*B2 project "*Development and Validation of Higher Order Structure Measurement Methods to Support ICH Guidelines*" and will be published on the National Physical Laboratory web-site when they are available. Validation data for this will also be published.

2.3. Suggested test methods

We tentatively suggest the methods below as candidate tests for the generation of in-house data sets. The threshold values will be product-specific and should be determined by users based on their individual requirements and the limits of the measurement. Detailed experimental protocols for these procedures and appropriate validation will be published on the national Physical Laboratory web site when they are available.

1. The combination of procedures should be capable of distinguishing in a statistically significant way between two samples of the same protein, which differ in concentration by a small amount (e.g. 2%).

2. The combination of procedures should be capable of distinguishing between the circular dichroism spectrum of an appropriate reference protein and the same protein "spiked" with a specified amount of a specified impurity protein (e.g. 5% w/w of commercially available lysozyme spiked into commercially available bovine α_1-acid glycoprotein).

3. The combination of procedures should be capable of distinguishing between the spectra of the same sample before and after a deliberate change in the wavelength scale, e.g. by 0.2 nm.

3. Recommendations for obtaining and comparing CD spectra

In this section we define the major sources of error in obtaining CD spectra and outline means to reduce their impact, and suggest experimental means to assess their impact on the overall process. We also provide an overview of the steps necessary in data analysis and what it should be intended to achieve.

3.1. Environmental considerations

All sophisticated scientific instruments perform better if maintained under stable and appropriate environmental conditions, particularly if they are being called upon to measure small differences with great accuracy. CD instruments are no different.

Source of error	Effect	Recommendations
Temperature changes – instrument effects.	These cause changes in electronic components, which often translate as baseline drift, but could also affect wavelength and ellipticity calibration.	1
Temperature changes – sample effects	Unless the temperature of the sample is separately controlled, temperature changes will influence cell path length, protein concentration (through expansion) and the inherent CD of the protein.	1, 27
High and/or variable humidity and temperature.	May increase the rate of degradation of the instrument or its optics	1
Vibration	Damaging to the optics and their calibration	2

Table 3 Errors arising from environmental factors

Recommendations

1. The instrument should be located in an air-conditioned laboratory maintained at a stable temperature. Optimal temperature and humidity conditions for each instrument will be given in the information provided by the manufacturers. Draughts and sources of local heating (including sunlight) and vibration should be avoided.
2. The instrument should be maintained in a mechanically stable environment, free from vibration or shocks. After moving the instrument, it should be tested thoroughly and serviced if necessary.
3. There should be access to a source of purified water (double distilled or equivalent) for preparation of buffers etc.
4. The lamps of some instruments require cooling water, which may come from a tap or from a recirculating chilling system.

3.2. The CD instrument

CD instruments benefit from regular maintenance and re-calibration. The two factors that require calibration are wavelength and sensitivity. A full discussion of the use of chemical reference materials for ellipticity and wavelength calibration is included in Appendix B, and the variation between instruments in the UK is covered in Appendix A. In general terms, modern instruments are more stable than older instruments, and the frequency of calibration is less. Another factor is drift of the ellipticity baseline with time, and this can happen over relatively short time scales. The rate at which this happens for particular instruments in specific environmental conditions should be assessed for each instrument, and from these data an appropriate regime for measuring the baselines developed.

Source of error	Effect	Recommendations
Incorrect wavelength calibration (offset)	Can be corrected during instrument servicing. It may not be a problem if all data is collected on one instrument, but the error can change as a result of instrument servicing.	6, 7
Incorrect wavelength calibration (non-linearity)	Likely to be a property of the instrument design and probably unchangeable. It may not be important if all data are collected on one instrument	6, 7
Incorrect calibration of ellipticity measurement	A key calibration step for the instrument – see Appendix B	6, 9, 10, 11
Nitrogen flow, impure nitrogen	Potential damage to optics and absorbance at short wavelengths	13, 14
Degraded or dirty optics	Leads to poor signal-to-noise. Should be observable from spectral changes when routine calibration is carried out, and corrected during servicing. Replacement optics may be required.	12
Short term changes in calibration	Considered to be those arising within a single day's work. These may arise from changes in the environmental conditions within the room during the day, or from instrument warm up.	1, 5
Long-term changes in calibration	Those that occur slowly between instrument recalibrations.	6
Changes in ellipticity offset over short time periods	This is observable from differences between baselines collected at regular intervals. The rate at which this occurs defines how often baseline spectra should be collected.	8

Table 4 Instrument derived sources of error

Recommendations

5. Allow the instrument to warm up to an equilibrium state before collecting data (approximately 45 minutes to one hour).
6. Have a regular maintenance and recalibration schedule which checks wavelength and ellipticity.
7. Undertake wavelength calibration using, for example, a neodymium or holmium filter, and optimise this for the region of greatest value for samples of interest (typically near or far UV CD for protein samples).
8. Measure the baseline drift for the instrument over the course of a working day, and from this design an appropriate schedule for the work. This might be to collect a baseline at the beginning and end of the day for a modern instrument, or more frequently for an older instrument.
9. Recommendations for the calibration of ellipticity using chemical calibrants are given in Appendix B.
10. The ellipticity of an ACS solution changes significantly with temperature (approximately 4% between 5 and 40 °C), and so a calibration solution stored at 4 °C must be allowed to equilibrate before use.
11. Although traditionally the ellipticity calibration has been carried out at a single wavelength, it is probably not independent of wavelength. Thus the optimal solution would be intensity calibrations at multiple wavelengths and application of a non-linear correction. However development of appropriate standards and calibration techniques are not yet available.
12. Monitor the signal-to-noise ratio or the HT voltage in a reference sample (which may be the calibrant used for ellipticity calibration) to monitor degradation of the optics of the instrument.
13. The CD instrument will require a relatively high flow of high purity nitrogen to purge the optics. Whilst cylinders can be used, in our experience they require changing too frequently for comfort and nitrogen from liquid nitrogen boil-off systems is preferable.
14. Unless specific means to vent the nitrogen from the instrument are in place, the laboratory may require alarm systems that indicate low oxygen levels. Depletion of oxygen by nitrogen replacement does not give rise to a physical symptom that warns the scientist in the laboratory.

3.3. The cell

The sample for analysis is inevitably enclosed in a cell, which is almost invariably made of quartz. Even the most perfect cell will have an absorbance and may have slightly different properties for the left- and right-handed circularly polarised light. The faces of the cell should be parallel and aligned perpendicularly to the light beam. The separation of the cell faces – the path length – will typically be short and experience shows that differences between the actual and nominal path length can be very significant.

Source of error	Effect	Recommendations
Cell produces a significant baseline	The more intense the cell baseline, the more critical is the subtraction of the blank from the test spectrum, and the more critical is the consistency of the orientation in the cell holder	17, 18, 20, 33
Cell not clean	Absorbance will reduce S/N in the final spectrum and limit the wavelength range over which data can be collected. The blank spectrum measured with buffer may not be an exact match of the cell blank in the test spectrum, leading to errors after subtraction.	19
Inconsistent placement of cell in instrument	Cells have a significant ellipticity at certain wavelengths and if the orientation changes between the blank and test spectrum, the subtraction may not appropriately correct the spectrum of the sample.	18
Cell path length not as reported	Practically equivalent to error in protein concentration	15, 16

Table 5 Cell derived sources of error

Recommendations

15. The path length of the cell should be determined by an independent means and used to correct the experimental data. The true path length of the cell tends to have greater proportional error for short path length cells (used to obtain far UV CD spectra), but can also be significant for 1 cm cells.
16. Appropriate means for measuring the path length include interferometry (only suitable for short path length cells), optical absorption measurements using a reference compound such as potassium dichromate, or by measurement of the ellipticity of an optically active reference compound such as ACS.
17. The baseline and the experimental data should be collected using the same cell.
18. A protocol to allow reproducible positioning of the cell in the holder, and within the light path, should be developed. The orientation of the cell should be the same each time, aided by the markings on the cell.
19. The cell should be cleaned with appropriate detergents (e.g. Hellmanex) at regular intervals, and should the baseline show evidence that the cell is not clean (e.g. the blank has a low intensity “protein” spectrum arising from adsorbed protein), the cell should be cleaned until this baseline disappears.
20. High UV absorbance of the cell, even without a CD component, will lead to reduced signal-to-noise in the spectrum and probably limit the wavelength range over which data can be collected. Cells showing a high absorbance should be avoided. Similarly, cells showing a high baseline in CD should also be avoided. This is usually due to residual strain in the cells, which may be removed by annealing.

3.4. The sample

Source of error	Effect	Recommendations
Incorrect determination of protein concentration	Creates a comparability error when collecting data from multiple samples, which is similar in type to errors in cell path length or calibration of ellipticity	21
Particulate matter leading to light scattering	This leads to artefacts related to the "particle" rather than the molecule, which occur at wavelengths where no absorption occurs and the magnitude of which depend on the distance between the sample and the detector.	24, 18
Absorbing buffers leading to poor signal-to-noise	These decrease the S/N of the spectrum and limit the wavelength range over which data can be collected.	26
High concentrations leading to poor signal-to-noise	The S/N of the CD data is reduced at high absorbance, which limits the accuracy with which spectra can be compared.	22, 23
Low concentrations leading to poor signal-to-noise	Weak signals from the test sample reduce signal to noise and the relative importance of errors in background subtraction, increasing the variability of the "compliant" spectra.	22
Chiral, absorbing excipients (e.g. sugars)	The spectrum is then an overlay of two spectra, and correction for e.g. protein concentration may cause additional problems. Also limits S/N in key areas because the excipient will be absorbing.	26
Inconsistent pH of buffers	The spectrum of the test sample may vary slightly with pH or temperature, leading to greater variability between "compliant" spectra and reduced sensitivity to "significant" spectral differences.	25
Sample temperature changes between runs	The spectrum may change slightly with temperature, increasing "compliant" spectra variability and reducing the ability to detect changes. Temperature changes affect slightly the cell path length and sample concentration (due to expansion of the solution).	1, 27

Table 6 Sample derived sources of error

Recommendations

21. Ensure as far as possible that all test samples are at the same protein concentration. A reliable and accurate method for the determination of protein concentration should be employed.
22. The sample concentration should be as high as possible, but the absorbance[3] of the sample should not exceed 1. For pharmaceutical products being analysed with minimal processing, control of the absorbance of the sample is likely to be by appropriate choice of the path length of the CD cell.
23. The concentration should be such that the Beer-Lambert law is obeyed.
24. Samples should be free from dust and other particulates (including aggregates). Sample should be filtered before spectral acquisition whenever feasible.
25. Check the pH of the buffer.
26. Where possible, the sample should be in a non-UV-absorbing buffer and free from chiral excipients such as sugars, although often this will not be in the control of the spectroscopist.
27. Sample temperature should be allowed to equilibrate before measurement. This may be to the ambient temperature or using a temperature control accessory within the instrument.

3.5. Data collection parameters

Choice of inappropriate parameters can lead to production of a spectrum that is erroneous, either due to errors in the wavelength and intensities at peak maxima, or the points at which the trace crosses zero. The most likely cause of such artefacts is the use of a too rapid scan speed, a long “time constant”, “response time” or “integration time”, or an inappropriately broad spectral band width (which is related to the slit width). On the other hand, use of too narrow a slit width or band width results in little light passing through the sample and poor signal-to-noise. Because different manufacturers define parameters in different ways (or change the definition of terms between models), or collect spectra in different ways, this choice of appropriate parameters depends in part on a knowledge of the spectropolarimeter being used. In a study of the effect of small changes in scan parameters, we found that within a broad range of “appropriate” parameters, the spectra collected in a fixed amount of time are similar in appearance and signal-to-noise.

[3] On theoretical grounds, the optimum absorbance for CD is 0.869 (see Johnson, W.C., Jr., Circular Dichroism Instrumentation, in “Circular Dichroism and the Conformational Analysis of Biomolecules”, G.D. Fasman, Editor. 1996, Plenum Press: New York. p. 635-652.

Source of error	Effect	Recommendations
Scanning the spectrum too quickly	Errors in peak maxima and peak intensities, with a shift to short wavelength and reduced intensity with fast scanning speeds	28
Inappropriately long time constant	Errors in peak maxima and peak intensities, as the spectrum is being averaged as the wavelength is scanned	28
Inappropriately broad bandwidth/slit width	Errors in peak maxima and peak intensities	29, 30
Inappropriately narrow bandwidth/slit width	Poor signal-to-noise due to too little light passing through the sample, which can be rectified by accumulating scans.	29, 30

Table 7 Errors arising from poor choice of data collection parameters

Recommendations

28. The scan rate should be set at such a value that the product of the scan rate (in nm s^{-1}) and the time constant (in seconds) should be less than the bandwidth in nanometres. The exact application of this rule will depend upon the manner in which data is collected and the definition of the terms - "time constant", "response time" or "integration time" - used.
29. Spectral bandwidths/slit widths should be kept below 2 nm to reduce errors below 1%. Typical slit widths are 1 nm for normal work and 0.1 nm for high-resolution work.
30. Data pitch (or step size) – the wavelength difference between data points - should be sufficiently close to properly define a peak. The data pitch should be less than 10% of the full width half height (FWHH) of a spectral peak.
31. The signal-to-noise ratio increases with the square root of the number of scans, whilst the time taken increases linearly: our experience has been that within the recommended data collection parameters, spectra collected using different parameters, but taking the same amount of time, are closely comparable.
32. The spectral width should contain a region of at least 20 nm (at long wavelength) with minimal signal, so that the expected spectrum lies very close to the baseline. This allows the accuracy of the baseline subtraction and spectral drift to be assessed.

The following equation usefully summarises recommendations 28, 29 and 30:

$$\frac{S \cdot \tau}{60} < B < \frac{W}{10}$$

Equation 1 Selection of optimal scan parameters for CD

***S* is the scan speed in nm·min^{-1}; τ is the response time in s; *B* is the spectral bandwidth in nm; and *W* is the width of the spectral features in nm.**

3.6. Data pre-processing

There may be times when pre-processing of the data is appropriate to improve the comparability of data collected on the same instrument at different times, on different instruments or in different cells. For example, the sample intensity may be scaled to account for errors in protein concentration, changes in calibration, or differences in the path length of the cell. Similarly, it may become apparent from running standard spectra (e.g. the ACS calibrant, neodymium or holmium filters) that wavelength calibration is changing with time or as a result of servicing and recalibration. At that stage a linear or non-linear correction can be applied to the wavelength axis.

In other cases it may be appropriate to improve signal to noise or remove high frequency noise superimposed on relatively broad CD signals. There are a number of mathematical approaches that allow this to be done, including Savitzky-Golay and Fourier transform approaches.

Baseline correction can also be considered a form of data pre-processing, and is absolutely crucial to the production of comparable spectra. Experience suggests that the baseline can drift significantly on a relatively short timescale, and it may be necessary to measure multiple baselines within a working day, and the baseline subtracted from the test spectra should be a combination of these. Modern instruments tend to be more stable. For a detailed discussion of pre-processing, please see Appendix C.

Source of error	Effect	Recommendations
Over-aggressive smoothing of data	Risks removing real spectral feature, particularly sharp features	36
Spectral manipulation approaches change the spectrum and complicate comparison.	Reduced data comparability	37

Table 8 Errors arising during data pre-processing

Recommendations

33. Subtraction of the cell baseline should be carried out. The frequency at which the baseline is re-measured and how to combine multiple baselines collected on the same day (due to relatively fast drift) should be validated and defined, as it is likely to depend upon the instrument being used.
34. Noisy data, usually as a result of high absorbance, collected at short wavelengths should be discarded before passing the data set for analysis and data comparison.
35. If other forms of data pre-processing can be avoided, they should be.
36. Data smoothing should be validated to ensure that it does not change peak maxima or peak intensities. Fitting procedures can be valuable for accurate determination of peak maxima.

37. The same methodology for data smoothing should be applied to all samples and reference data sets. The approaches used should be validated with reference data sets to assess how they affect spectral comparison.
38. The corrections applied should be based on independent data sets (e.g. wavelength shifts determined from the spectra of neodymium or holmium filters) rather than the data of the test and reference samples. Similarly, intensity variation due to protein concentration differences and/or the path length of the cell should be based, whenever possible, on external measurements.

3.7. Data analysis and comparison

Within the context of a generic quality control method, the need is to show that the complete process is sufficiently precise to distinguish, at a statistically significant level, the spectra from "non-compliant" and "compliant" batches. A good method should be able to distinguish spectral changes associated with a number of different reasons from the sample being "non-compliant", such as, for example, partial thermal denaturation or buffer pH. This is a spectroscopic and statistical problem, and does not address the problem of whether, for that particular product, any or all of these causes of being "non-compliant" has a significant effect on biological activity, immunogenicity of product safety. As such, the validation of the data analysis and comparison package can be divorced from the final product to which it will be applied.

To be an objective assay, the data analysis needs to avoid visual comparison of spectra. Similarly, for an approach based on, for example, cluster analysis, the decision should not rely on a visual comparison of whether two clusters are the same or different. The output from the data analysis and comparison package should be expected to be a single number which represents the probability that the spectrum (or spectra) of the sample is different from that of the normal sample(s). It would then be possible to compare this with a numerical specification.

For some products, it is conceivable that there are a number of different clusters for "compliant" samples (where small structural changes observable by CD do not significant change biological activity, immunogenicity or product safety, as indicated by other assays), and so the comparison may need to be with several "compliant" datasets. Similarly, where there are a number of "non-compliant" clusters, it may be useful to obtain some indication of the reasons for the failure of a particular sample.

For a detailed discussion of data analysis approaches, please see Appendix C.

Recommendations

39. The data comparison approach chosen should return a single number, which indicates the likelihood that the test sample is different from the reference "compliant" sample or samples.
40. Methodology that can distinguish "abnormal" spectra arising from the test sample due to different forms of denaturation or abuse should be favoured.
41. The sensitivity of the data comparison method to distinguish slightly different spectra should be assessed using reference data sets or reference samples prepared by mildly aggressive treatment.

42. The relevance of spectral differences to the biological activity of the product needs to be assessed on a product-specific basis, and this will determine how a specification is set for that product.