Validation of a concentration assay using Biacore C

- Guideline for development of a GxP - compliant concentration assay
- Support for informed decision-making in drug development

Introduction

In the process of measuring active concentrations of pharmaceuticals, high demands are placed on the quality of the data on which any decision to proceed to clinical trials will be based. Data gathered using any technology must be accurate and precise. Method validation (defining the acceptable limits within which data is deemed reliable) is therefore a crucial part of the overall process through which a prospective drug passes before it is tested in clinical trials. Samples for pharmaceutical concentration measurements are also taken from reactor runs, to monitor the development of purification processes, in stability studies and in hybridoma screening. Here we present the results obtained during a validation process within the pharmaceutical industry for a concentration assay using Biacore™ C system. The results confirm that the quality of performance of this assay was sufficiently high to meet GxP demands placed on drug companies by regulatory authorities.

Biacore C is specifically designed for concentration analysis of pharmaceuticals. The instrument software supports GxP-regulated working processes with user-friendly wizards for assay development, immobilization strategies and data analysis. In addition, as current regulations demand restricted access to data generated by analytical instruments, Biacore C software makes it possible to lock and fix assay templates.

In the example cited here a receptor/ligand system was investigated. Both receptor and ligand are proteins. The receptor protein was immobilized at high density on the surface of an activated sensor chip. Reference material, quality control (QC) samples and test samples were then diluted and injected over the receptor surface to generate sensorgrams in which binding data is recorded over real time. The initial binding rate of the ligand to immobilized receptor (sensorgram slope) was measured under conditions of limiting mass transfer (diffusion). The rate, expressed as resonance units per second (RU/s) correlated directly with active ligand concentration. The binding rates of the samples were converted into active concentration using the reference standard curve where the binding rate of reference material was plotted against the concentration. The final results were expressed either as percent binding of sample relative to reference material or as a concentration.

Concentration assay validation: the cornerstones

Several criteria were tested over the course of the validation outlined below. Experiments should include, but are not necessarily limited to the following tests:

Parameter	Question addressed
Specificity	To what extent does the assay cross-react with related targets?
Linearity, range, detection and quantitation limits	To what degree of certainty can concentration measurements be interpolated on a standard curve and over what range can concentration measurements be confidently stated?
Accuracy	Do the determined concentration measurements lie within acceptable error limits when compared with nominal concentrations?
Precision	Can similar concentrations be measured with an acceptable level of reproducibility between users, on different occasions (inter-assay variation) and within the same assay (intra-assay variation)?
Robustness	Is the assay robust enough to be performed under sub-optimal conditions e.g. after repeated freeze/thaw cycles of the sample or if an alternative buffer system is used?

Acknowledgement

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Recommended steps in concentration assay validation

The steps outlined below are general guidelines for validating a novel concentration assay. The data presented are drawn from a specific assay validation carried out at Bristol-Myers Squibb Inc. in Hopewell, New Jersey. Before the validation process was started, a validation protocol was written.

Guidelines provided by the CDER, CBER, the "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)" and company internal Standard Operation Procedures derived from the guidelines were used to determine the content of the validation protocol. The protocol outlined the validation experiments that were to be performed and included expected results and acceptance criteria for each parameter investigated. After performing the experiments and data evaluation, the results were summarized in a Validation Report. This final report contains all important information on the validated assay. It is required for each analytical method that is used to test commercial drugs and usually will be reviewed by regulatory agencies.

Results

Specificity

Selected compounds that were predicted to cross react with the ligand were analyzed. Compounds included those that were structurally related to the ligand or receptor, or that were present in buffers during the ligand purification process. The following samples were investigated:

Test compounds and specificity control procedures:

- The sample: a protein was diluted to 2 µg/ml and analyzed as sample binding to receptor.
- Analyte specificity control: a protein structurally related to the ligand that was expected to bind to the receptor. It was diluted to 2 µg/ml and analyzed as sample binding to receptor.
- Receptor specificity control: a protein structurally related to the receptor was immobilized on a sensor chip surface and ligand at 2 µg/ml was injected over that surface and analyzed as sample binding to receptor.
- Host cell proteins (HCP) present during the purification of ligand may bind to receptor. HCP were diluted to 2 µg/ml and analyzed as sample binding to receptor.

Table 1 summarizes the results from these specificity experiments. No specific cross-reactivity was detected except for specificity control protein.

Table 1. Specificity of analyte-binding to specific and non-specific human receptors. Neither the specificity control antibody nor HCP bound to specific receptor. None of the compounds tested bound to non-specific control receptor.

Sample	Binding to specific receptor	Binding to non-specific receptor
HCP	-2 RU	-3 RU
Analyte	228 RU	-2 RU
Specificity control protein	268 RU	-2 RU

Linearity and range

Linearity and range were assessed in eight independent runs performed by two analysts. Each run consisted of a standard curve covering a wide concentration range. Each concentration was prepared in triplicate. The relationship between instrument response (slope in RU/s) and concentration of ligand (in ng/ml) was established by statistical evaluation and was best described by a four-parameter logistic regression function. For both analysts, the overall accuracy and precision for the standard concentrations were within 10% (% deviation from nominal and % CV) over the range from 250 ng/ml to 16 µg/ml (Figure 1).

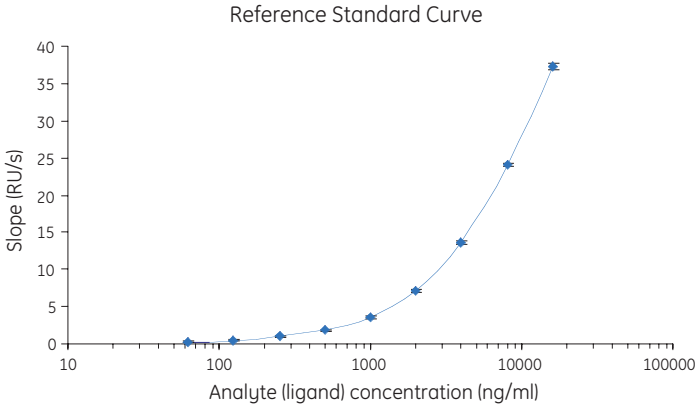

Figure 1. Linearity and range limits. The Linearity of the standard curve was statistically evaluated in the validation experiments. A four parameter curve fit function to describe best the relationship between log analyte concentration and binding slope with R^2 always > 0.99 was determined. LOD and LOQ were determined to be 62.5 and 250 ng/ml, respectively. Slope values for reference concentrations are shown here for all experiments performed (n = 56) for one drug candidate. In a typical experiment, n=3 for each concentration.

Detection and quantitation limits

Detection and quantitation limits (LOD and LOQ, respectively) were validated by analyzing triplicate samples in three independent runs at ligand concentrations of 15.6, 31.25, 62.5, 125, 187.5 and 250 ng/ml. LOD was defined as the lowest concentration of ligand that produced a mean response (slope) significantly higher than the mean response at 0 ng/ml. LOQ was defined as the lowest concentration of ligand that could be quantified with acceptable accuracy and precision (defined as SD within 20% of the mean value and CV at most 20%). Statistical analysis suggested that LOD was 62.5 ng/ml and that LOQ was 250 ng/ml, points at which both SD and CV for accuracy and precision were less than 10%.

Accuracy and precision

Accuracy and precision of the assay were evaluated with a set of three Quality Control (QC) samples. Accuracy was defined as the % deviation from nominal concentration. Precision is expressed as % CV (relative standard error). QC samples were prepared in HBS-EP buffer at the three target concentration levels of 300, 2500, and 4500 ng/ml and frozen in 300 µl aliquots at -80ºC. The sample concentrations were determined in three independent experiments and the average concentration results were reported as the "nominal" QC concentrations in a Certificate of Analysis for each sample. On the day of an experiment, one vial from each of the three samples was thawed at room temperature. The samples were placed in rack positions as described in the method wizard and analyzed in triplicate. Observations were made in triplicate at each of the QC concentration levels. A second investigator also analyzed the standard concentrations and quality controls three times. The results are presented in Table 2.

Table 2. Summary of accuracy and precision data for QC samples. n = 24.

	288 ng/ml	2387 ng/ml	4273 ng/ml
Accuracy:			
Mean	275	2356	4275
% deviation	-4.7%	-1.3%	0.05%
95% confidence levels	(266–284)	(2293–2419)	(4158–4392)
Precision (% CV):	6.8	5.5	5.6
Between-analyst:	8.4	6.7	6.9

Robustness

Sample at different concentrations was exposed to stress conditions and the accuracy of concentration measurements were compared with data taken under normal conditions. The effects of the following stress conditions on sample stability were investigated:

- Stock solutions of Analyte Reference material and QC samples were exposed to 25ºC for between 2 hours and 8 days and results were compared from samples standing at 4ºC for 1 day or 8 days. Analyte stock solution and samples were judged to yield acceptable, accurate concentration data after up to 1 day incubation at both 4°C and 25°C (Table 3). Acceptability was defined in the validation protocol and was set to ≤20% for precision (% CV) and accuracy (% deviation from nominal).

Table 3. Stability of reference and QC samples after protracted storage at 25°C and 4°C. nd = not determined.

Accuracy (% deviation from nominal)						
Concentration	Control	25°C for 2 hours	25°C for 1 day	25°C for 8 days	4°C for 1 day	4°C for 8 days
125 ng/ml	-12.0	-13.6	-13.6	-20.6	8.0	8.0
250 ng/ml	2.4	5.2	3.2	-1.6	-4.0	-5.2
500 ng/ml	2.8	2.2	4.8	8.4	-0.8	-0.6
1 µg/ml	0.0	-0.6	-1.0	1.0	-1.4	1.0
2 µg/ml	-1.0	-0.5	-0.5	-2.0	1.5	-0.5
4 µg/ml	0.5	0.3	0.3	0.8	-0.5	0.0
8 µg/ml	0.0	0.0	0.0	-0.1	0.0	0.0
QC 288 ng/ml	4.2	-2.1	-1.4	32.6	-5.6	nd
QC 2.387 µg/ml	1.8	1.4	4.3	19.8	-5.3	nd
QC 4.273 µg/ml	2.3	1.1	5.3	21.5	-1.7	nd

- Samples were subjected to one or several freeze/thaw cycles before concentration analysis. The accuracy of the assay was judged acceptable after three freeze/thaw cycles for the calibration samples and two for the QC samples (Table 4). Acceptability was defined in the validation protocol and was set to ≤20% for precision (% CV) and accuracy (% deviation from nominal).

Table 4. Stability of reference and QC samples after repeated freezing and thawing.

	Accuracy (% deviation)			
Concentration	Control	1 freeze/thaw cycle	2 freeze/thaw cycles	3 freeze/thaw cycles
125 ng/ml	-12.0	18.4	nd	nd
250 ng/ml	2.4	12.8	-12.0	16.0
500 ng/ml	2.8	14.0	-8.2	13.2
1 µg/ml	0.0	13.0	-10.3	6.0
2 µg/ml	-1.0	9.0	-8.0	0.0
4 µg/ml	0.5	10.5	-7.8	-0.8
8 µg/ml	0.0	nd	-7.8	-1.8
QC 288 ng/ml	4.2	-3.1	-0.3	-21.2
QC 2.387 µg/ml	1.8	1.8	0.5	-2.0
QC 4.273 µg/ml	2.3	-0.5	1.6	0.2

- Samples were injected over a range of receptor densities on the sensor chip surface. Between approximately 1000 and 10000 RU of receptor were immobilized and the accuracy and precision of the data at each concentration was calculated. Both assay accuracy and precision performance were optimal at receptor immobilization levels of between 3000 and 9000 RU (Figure 2).

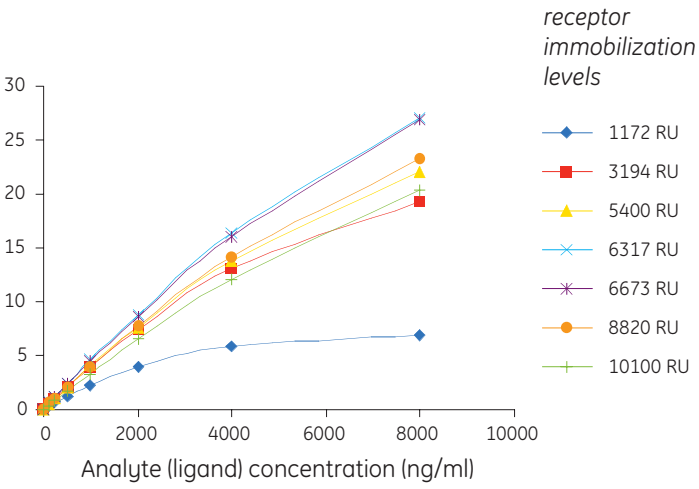

Figure 2. Influence of ligand density on assay performance.

- Ligand was injected at 2 µg/ml and the surface was regenerated using citrate buffer at pH 4.0, followed by an injection of water. The surface of the sensor chip was deemed to be stable and yielded consistent concentration data over at least 286 injections of sample (Figure 3 and Table 5).

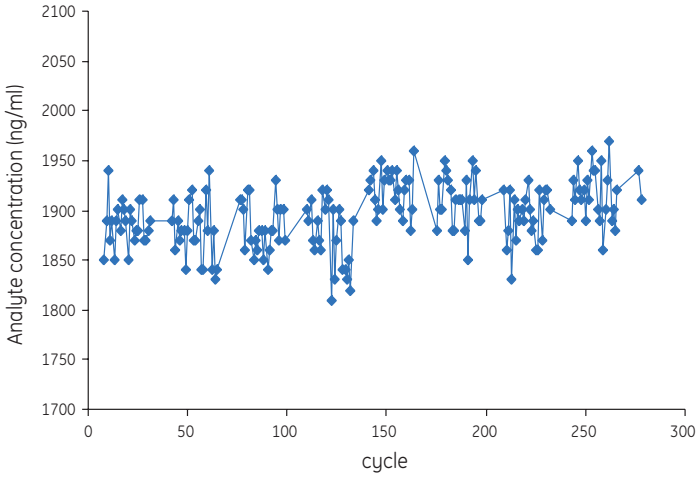

Figure 3. Test of receptor stability by repeated injection of analyte.

Table 5. Receptor stability over 286 repeated analyses.

Parameter	Slope (RU/s)	Concentration
Mean	6.94	1.895 µg/ml
SD	0.10	31 ng/ml
% CV	1.50	1.62
Minimum	6.62	1.81 µg/ml
Maximum	7.2	1.97 µg/ml
Minimum (% difference relative to mean)	-4.5	-4.5
Maximum (% difference relative to mean)	3.8	3.9
Mean of first ten samples	7.025	1.877 µg/ml
Mean of last ten samples	6.791	1.91 µg/ml
% change from first ten to last ten samples	-3.3	1.2

- Spike recovery was determined in different buffers and media that may be used during the production, processing, and manufacturing of the ligand, i.e. cell culture media, elution buffers from different column purification steps as well as formulation buffers. Spiked samples were recovered at between 91% and 119% of nominal values in all buffers tested (Table 6).

Table 6. Spike recovery (% nominal values) in selected buffers.

Buffer	Dilution 1:50	Dilution 1:100	Dilution 1:500
1	103	115	94
2	110	117	98
3	108	119	104
4	109	116	100
5	104	112	118
6	104	94	115
7	105	115	110
8	110	118	91

Conclusions

The validation of a concentration assay for any novel drug is an empirical process. The example given in this Application Note may serve as a guideline to investigators wishing to develop an accurate, precise and robust concentration assay that is fully GxP-compliant. Documented performance of any concentration assay is a mandatory regulatory requirement, and it is therefore worth investing time and care to ensure that the assay will live up to these demands.

The data from the assay validation procedure described here show that Biacore C supports concentration assays of sufficient quality to enable scientists to make informed decisions on taking a drug to clinical trials.

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