National Cancer Institute
Division of Cancer Prevention

Requirements for:

PHARMACOKINETIC AND BIOMARKER METHODS DEVELOPMENT

1.0 PHARMACOKINETIC AND BIOMARKER METHODS DEVELOPMENT AND VALIDATION REPORTS

All pharmacokinetic and biomarker laboratory techniques, assays, and procedures shall be developed and validated according to the parameters described in this section, and a draft report prepared for NCI, DCP approval. The approved Method Development and Validation Report will be incorporated into the contract Final Report. The format of this section should be followed when preparing Pharmacokinetic and Biomarker Methods Development and Validation Reports.

Summaries of specific recommendations for methods development and validation are presented in this section. In general, establish the following for each method:

- Conditions to ensure stability of the analyte or antigen in the matrix during collection, processing, storage and analysis.
- Linear range: The range over which the procedure has been demonstrated to produce a reproducible, linear response. (A reproducible, nonlinear response may also be acceptable.)
- Specificity: The ability of the method to measure only what it is intended to measure.
- Sensitivity: Limit of detection, limit of quantitation, and 95% confidence interval of standard curve.
- Inter-day and intra-day accuracy: The closeness of determined value to the true value.
- Inter-day and intra-day precision: The variability of replicate determinations.
- Ruggedness: Variability between operators, instrument, columns, *etc.*

Draft Methods Development and Validation Reports shall include all analytical and computational documentation resulting from these efforts. In addition, sample chromatograms or endpoint measurements from method development should be submitted; densitometric scans, photographs, *etc.,* may be presented as appropriate. These samples should include the following: blank plasma, urine, or tissue; analyte in buffer; and analyte in plasma, urine, and/or tissue.

When sample analysis is conducted at more than one site, it is necessary to validate the method at each site and provide appropriate validation information for different sites to establish interlaboratory reliability. Any modification of an analytical method would require revalidation of the procedure.

2.0 PHARMACOKINETIC ANALYTICAL METHODS DEVELOPMENT AND VALIDATION

Recommendations for pharmacokinetic methods development and validation are derived from the Conference Report *Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies* [1].

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples play a significant role in the evaluation and interpretation of pharmacokinetic data. It is essential to employ well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. Specific validation criteria are needed for methods intended for analysis of each analyte (drug and/or metabolite). Method validation includes all of the procedures required to demonstrate that a particular method for the quantitative determination of the concentration of an analyte is reliable for the intended application. The parameters essential to ensure the acceptability of the performance of an analytical method are stability of the analyte in the biological sample under study storage conditions, accuracy, precision, sensitivity, specificity (selectivity), response function, and reproducibility.

2.1 Specific Recommendations for Pharmacokinetic Analytical Method Development and Validation

2.1.1 A specific detailed description of the analytical method (standard operating procedure; SOP) must be prepared, including a statement of purpose, a listing of necessary reagents, test solutions and mixtures, and directions for preparation, storage, and expiration dating of required materials. It should also include a listing of the required instruments and instrumental parameters. Representative chromatograms and calculations, if used, should also be provided. The SOP should contain sufficient information to allow repetition of the method by a qualified investigator.

2.1.2 Each step of the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables, from the time of collection of the material up to and including the time of the analysis, may affect the estimation of analyte in the matrix. Whenever possible, the same biological material as that in the intended samples should be used for validation purposes. Variability of the matrix due to its physiological state, and the influence of

freeze-thaw cycles should be considered, if appropriate. The results of these investigations will define acceptable conditions and restrictions for collection, storage and analysis of samples; these conditions are then stated in the SOP.

2.1.3 The concentration range over which the analyte can be determined must be defined in the method, based on evaluation of actual standard samples, including their statistical variation. This defines the standard curve. The standard curve consists of at least five concentrations, excluding blanks, and should cover the entire range of expected concentrations. The simplest relationship for response as a function of concentration should be determined and the fit statistically tested; this relationship must be demonstrated to be continuous and reproducible. The equation of the function should be given and represented graphically.

2.1.4 The limit of detection (LOD) is the lowest concentration of an analyte that the method can reliably differentiate from background levels. The lower limit of quantitation (LOQ) is the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability, respectively. The LOQ is determined by using at least five samples independent of standards and by determining the coefficient of variation (CV) and/or appropriate confidence interval. The lower LOQ serves as the lowest concentration of the standard curve.

2.1.5 Accuracy and precision with which known concentrations of analyte in the biological matrix can be determined must be demonstrated. Within- and between-run accuracy and precision should be calculated with commonly accepted statistical procedures. Determination of accuracy and precision can be accomplished by analysis of replicate sets of analyte samples of known concentrations from an equivalent biological matrix, using a minimum of five determinations per concentration. Specific criteria must be set for accuracy and precision over the range of the standard curve. At least three concentrations representing the entire range of the calibration curve should be studied—one near the lower LOQ, one near the center, and one near the upper boundary of the standard curve. The mean value should be within ±15% of the actual value except at LOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% CV, except for LOQ, which should not exceed 20% CV.

2.1.6 If possible, the specificity of the assay methodology should be established using six independent sources of the same type of biological sample.

2.1.7 If there are intermediate steps between the biological matrix and the final assay, such as extraction of biological samples, and if parallel processed standards are not being used, recovery should be established and used to determine results.

2.1.8 It is suggested that validation include investigation of samples from dosed subjects. If possible, stability of the analyte in the matrix from dosed subjects should be confirmed.

2.2 Recommendations for Routine Application of Pharmacokinetic Methods

2.2.1 A standard curve should be generated for each analytical run for each analyte and should be used to calculate the concentration of analyte in unknown samples within that run.

2.2.2 Acceptance criteria for application of the assay and a protocol for repeat analysis should be established prior to sample analysis. Accuracy should be not more than 15% deviation from the nominal value and precision should be not more than 15% CV. At the LOQ, 20% is acceptable for both precision and accuracy.

2.2.3 Quality-control (QC) samples are matrix spiked with analyte. QC samples in duplicate at three concentrations (one near the LOQ, one in midrange, and one approaching the high end of the range) should be incorporated into each run. Results of QC samples provide the basis for accepting or rejecting the run.

2.2.4 Estimating unknowns by extrapolations below the low standard or above the high standard should be avoided. Instead, the standard curve should be redetermined or samples reassayed after dilution.

3.0 BIOMARKERS METHODS DEVELOPMENT AND VALIDATION

For biomarker assays in general, the recommendations in 1.0 should be followed. Though many types of assays exist, only immunohistochemical is specifically discussed. Immunohistochemistry is used to characterize a wide range of biomarkers. This technique is not linear, it saturates at higher intensities of staining, and is accentuated at the lower intensities of staining. Dr. Grizzle has extensive experience with immunohistochemical techniques and has developed a semiquantitative index which incorporates both the intensity of staining of individual cells and the percent of cells staining at each intensity [2]. The parameters essential to ensure the acceptability of this technique include determining stability of the antigen in the biological sample (*e.g.*, tissue biopsy) under study storage conditions, sensitivity of the assay, specificity of the antibody, and intra- and inter-assay reproducibility.

3.1 Specific Recommendations for Immunohistochemical Methods Validation

3.1.1 A specific detailed description and protocol of analytical method and scoring methodology (standard operating procedure) includes a statement of the method, a listing of necessary reagents, test solutions and mixtures with directions for their preparation, storage conditions and usable shelf life specified. It should also include a listing of the required instruments and instrumental parameters, which contains sufficient

information to allow repetition of the method by a qualified investigator.

3.1.2 Stability of the antigen in the biological sample is determined under identical study storage conditions (*e.g*., temperature, duration) and sample conditions (*e.g*., frozen tissue biopsy, paraffin-embedded tissue).

3.1.3 Sensitivity of the assay should be determined using at least three sequential antibody dilutions.

3.1.4 Specificity of the assay should be determined by using at least two different antibodies to the same antigen, if feasible. It is also important to perform the assay in the absence of antibody in order to determine nonspecific staining.

3.1.5 Intra-assay reproducibility should be determined by staining different serial sections from the same biological sample and/or by showing that different antibodies to the same antigen give similar patterns and intensities of staining.

3.1.6 It is necessary to purchase a sufficient amount of reagents (*e.g*., antibodies) with the same lot number to complete the study.

3.1.7 Inter-assay reproducibility should be determined by staining the same tissue sections for the same antibodies during multiple different assay runs on different days. For example, Dr. Grizzle demonstrated reproducibility by staining seven biological samples with three dilutions of various antibodies of interest. The staining was repeated four times each on different days and the results were analyzed by a qualified investigator without knowledge of the stain, concentration, or repeat status of the stain. Several parameters can influence reproducibility. First, variability in antigen expression within the tissue can be minimized by using adjacent sections. Second, day to day variability due to differences in staining procedure and conditions can be minimized by using an automated stainer.

Third, variability introduced by the evaluator can be minimized by averaging the scores of two or more observers.

4.0 REFERENCES

1. Shah, V.P., Midha, K.K., Dighe, S., McGilveray, I.J., Skelly, J.P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowall, R.D., Pittman, K.A., and Spector, S. Analytical methods validation: Bioavailability, bioequivalence and pharmacokinetic studies. *Pharm. Res.* 9: 588–592, 1992.

2. Myers, R.B., Lampejo, O., Herrera, G.A., Srivastava, S., Oelschlager, D., Brown, D.A.,

Waterbor, J.W., Grizzle, W.E. TGFá expression is a relatively late event in the progression of prostatic adenocarcinoma. *J. Urol. Pathol.* 3: 195–203, 1995.