

Center for Drug Evaluation
and Research (CDER)

Reviewer Guidance

Validation of Chromatographic Methods

November 1994
CMC 3

TABLE OF CONTENTS

I.	INTRODUCTION	1
II.	TYPES OF CHROMATOGRAPHY	2
A.	High Performance Liquid Chromatography (HPLC)	2
1.	Chiral Chromatography	2
2.	Ion-exchange Chromatography	3
3.	Ion-pair/Affinity Chromatography	3
4.	Normal Phase Chromatography	3
5.	Reversed Phase Chromatography	3
6.	Size Exclusion Chromatography	4
B.	Gas Chromatography (GC)	4
C.	Thin-Layer Chromatography (TLC)	5
III.	REFERENCE STANDARDS	5
IV.	PARAMETERS FOR VALIDATION OF HPL CHROMATOGRAPHIC METHODS FOR DRUG SUBSTANCE AND DRUG PRODUCT	7
A.	Accuracy	8
B.	Detection Limit and Quantitation Limit	8
C.	Linearity	11
D.	Precision	13
1.	Repeatability	13
a.	Injection Repeatability	13
b.	Analysis Repeatability	15

2.	Intermediate Precision	15
3.	Reproducibility	16
E.	Range	16
F.	Recovery	16
G.	Robustness	16
H.	Sample Solution Stability	17
I.	Specificity/selectivity	17
J.	System Suitability Specifications and Tests	21
1.	Capacity factor	22
2.	Precision/Injection repeatability	22
3.	Relative retention	22
4.	Resolution	22
5.	Tailing factor	23
6.	Theoretical plate number	26
K.	General Points to Consider	28
V.	COMMENTS AND CONCLUSIONS	29
VI.	ACKNOWLEDGEMENTS	29
VII.	REFERENCES	29

REVIEWER GUIDANCE¹

VALIDATION OF CHROMATOGRAPHIC METHODS

I. INTRODUCTION

The purpose of this technical review guide is to present the issues to consider when evaluating chromatographic test methods from a regulatory perspective. The document discusses the points to note and weaknesses of chromatography so that CDER reviewers can ensure that the method's performance claims are properly evaluated, and that sufficient information is available for the field chemist to assess the method. Analytical terms, as defined by the International Conference of Harmonization (ICH), 1993, have been incorporated in this guide.

Chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products and compounds in biological fluids. The components monitored include chiral or achiral drug, process impurities, residual solvents, excipients such as preservatives, degradation products, extractables and leachables from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites.

The objective of a test method is to generate reliable and accurate data regardless of whether it is for acceptance, release, stability or pharmacokinetics study. Data are generated for the qualitative and quantitative testing during development and post-approval of the drug products. The testing includes the acceptance of raw materials, release of the drug substances and products, in-process testing for quality assurance, and establishment of the expiration dating period.

Validation of a method is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose. Data thus

¹This guidance has been prepared by the Analytical Methods Technical Committee of the Chemistry Manufacturing Controls Coordinating Committee (CMC CC) of the Center for Drug Evaluation and Research at the Food and Drug Administration. Although this guidance does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the validation of chromatographic methods. For additional copies of this guidance, contact the Division of Communications Management, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-594-1012). Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet the World Wide Web (WWW) (connect to the FDA Home Page at WWW.FDA.GOV/CDER and go to the "Regulatory Guidance" section).

generated become part of the methods validation package submitted to CDER.

Methods validation should not be a one-time situation to fulfil Agency filing requirements, but the methods should be validated and also designed by the developer or user to ensure ruggedness or robustness. Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data that are generated for acceptance, release, stability, or pharmacokinetics will only be trustworthy if the methods used to generate the data are reliable. The process of validation and method design also should be early in the development cycle before important data are generated. Validation should be on-going in the form of re-validation with method changes.

II. TYPES OF CHROMATOGRAPHY

Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption-desorption steps on the stationary phase.

A. High Performance Liquid Chromatography (HPLC)

HPL chromatographic separation is based on interaction and differential partition of the sample between the mobile liquid phase and the stationary phase. The commonly used chromatographic methods can be roughly divided into the following groups, not necessarily in order of importance:

1. Chiral
2. Ion--exchange
3. Ion--pair/affinity
4. Normal phase
5. Reversed phase
6. Size exclusion

1. Chiral Chromatography

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on achiral stationary phases. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

2. Ion-exchange Chromatography

Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion (X^-), or cation exchange for sample positive ion (X^+). Gradient elution by pH is common.

3. Ion-pair/Affinity Chromatography

Separation is based on a chemical interaction specific to the target species. The more popular reversed phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s). Affinity chromatography, common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix) which interacts with its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

4. Normal Phase Chromatography

Normal phase chromatography is a chromatographic technique that uses organic solvents for the mobile phase and a polar stationary phase. Here, the less polar components elute faster than the more polar components.

5. Reversed Phase Chromatography

The test method most commonly submitted to CDER is the reversed phase HPLC method. UV detection is the most common detection technique.

Reversed phase chromatography, a bonded phase chromatographic technique, uses water as the base solvent. Separation based on solvent strength and selectivity also may be affected by column temperature and pH. In general, the more polar components elute faster than the less polar components.

UV detection can be used with all chromatographic techniques. The concern for this type of detector is the loss of sensitivity with lamp aging, and varying sensitivity at the low level depending on design and/or manufacturer. A point to note is that observations on the HPL chromatograms, by UV detection in combination with reversed-phase HPLC, may not be a true indication of the facts for

the following reasons:

- Compounds much more polar than the compound of interest may be masked (elute together) in the solvent front/void volume.
- Compounds very less polar than the analyte may elute either late during the chromatographic run or are retained in the column.
- Compounds with lower UV extinction coefficients or different wavelength maxima may not be detectable at the low level relative to the visibility of the analyte since only one wavelength is normally monitored.

6. Size Exclusion Chromatography

Also known as gel permeation or filtration, separation is based on the molecular size or hydrodynamic volume of the components. Molecules that are too large for the pores of the porous packing material on the column elute first, small molecules that enter the pores elute last, and the elution rates of the rest depend on their relative sizes.

B. Gas Chromatography (GC)

Gas chromatography is based on the volatilized sample transported by the carrier gas as the moving phase through the stationary phase of the column where separation takes place by the sorption/desorption process.

Samples for gas chromatographic analysis are normally low molecular weight compounds that are volatile and stable at high temperature. In this respect, residual solvents in drug substances and drug products are suitable for gas chromatographic analysis. Chemical derivatives can also be formed to achieve volatility and thermal stability.

Common detectors are flame ionization (FID) for carbon-containing compounds, electron capture (ECD) for halogenated compounds, flame photometric (FPD) for compounds containing sulphur or phosphorous and nitrogen-phosphorous (NPD) for compounds containing nitrogen or phosphorous. Chiral separation also can be achieved by gas chromatography. Separation by the packed column is rapidly being replaced by the capillary column that provides improved resolution and analysis speed. The location of the analyte on the gas chromatogram is

described by retention time (R_t) which is similar to HPLC.

C. Thin-Layer Chromatography (TLC)

Thin-layer chromatography is the simplest of the more common chromatographic techniques. Separation is based on migration of the sample spotted on a coated (stationary phase) plate with one edge dipped in a mixture of solvents (mobile phase). The whole system is contained in an enclosed tank.

Detection techniques include fluorescence, UV and sprays (universal and specific) for compounds that are not naturally colored. The location of the analyte on the TLC plate is described by the R_f value which is the ratio of the migration distance of the compound of interest to the mobile phase front.

Of the three techniques, gas, liquid and thin-layer, TLC is the most universal test method as all components are present on the plate and with appropriate detection techniques, all components can be observed. However, it normally is not as accurate or sensitive as HPLC. TLC has a higher analytical variation than HPLC, although one sees the "whole picture" when appropriate detection schemes are selected.

III. REFERENCE STANDARDS

A reference standard is a highly purified compound that is well characterized. Chromatographic methods rely heavily on a reference standard to provide accurate data. Therefore the quality and purity of the reference standard is very important. Two types of reference standards, chemical and nuclidic, exist. With the latter, the radio-label purity should also be considered as well as the chemical purity.

As described in the Guideline for Submitting Samples and Analytical Data for Methods Validation, the two categories of chemical reference standards are as follows:

- USP/NF reference standard that does not need characterization, and
- non-compendial standard that should be of the highest purity that can be obtained by reasonable effort and should be thoroughly characterized to assure its identity, strength, quality and purity.

The points to note are:

- Most USP/NF reference standards do not state the purity of the compound.

- The purity correction factor for non-USP reference standards is recommended to be included in the calculation of the test method.
- In addition to structurally-related impurities from the synthesis process, other process impurities like heavy metals, residual solvents, moisture (bound and unbound), pesticides for products of plant origin, and degradation products can also contribute to the lack of purity in the reference standard.
- The drying of the reference standard before use, if stated in the method, will eliminate residual solvent(s), unbound moisture and sometimes bound moisture (depending on the drying conditions). The drying step is always included for hygroscopic compounds. On the other hand, drying can result in the loss of a hydrate or cause degradation in heat-sensitive compounds.

Chromatographic test methods use either external or internal standards for quantitation.

- A. An external standard method is used when the standard is analyzed on a separate chromatogram from the sample. Quantitation is based on a comparison of the peak area/height (HPLC or GC) or spot intensity (TLC) of the sample to that of a reference standard of the analyte of interest.

The external standard method is more appropriate for samples as follows:

1. Sample with a single target concentration and narrow concentration range, e.g., acceptance and release tests.
2. Simple sample preparation procedure.
3. Increased baseline time for detection of potential extraneous peaks, e.g., impurities test.

- B. With an internal standard method, compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantitation is based on the response ratio of compound of interest to the internal standard vs the response ratio of a similar preparation of the reference standard (HPLC or GC). This technique is rarely used for TLC methods.

The internal standard method is more appropriate for samples as follows:

1. Complex sample preparation procedures, e.g., multiple extractions.
2. Low concentration sample (sensitivity being an issue), e.g., pharmacokinetics studies.
3. Wide range of concentrations expected in the sample for analysis, e.g., pharmacokinetics studies.

Although CDER does not specify whether the method must use an internal or external standard for quantitation, it is commonly observed that HPLC methods for release and stability and TLC methods use external standards; and methods for biological fluids and GC methods use internal standards.

The working concentration is the target concentration of the compound of interest as described in the method. Keeping the concentrations of the sample and the standard close to each other for the external standard method improves the accuracy of the method.

Recommendations:

1. Include the purity correction factor, if known, of the reference standard in the calculation.
2. State the working concentrations of the standard and sample in the method.

IV. PARAMETERS FOR VALIDATION OF HPL CHROMATOGRAPHIC METHODS FOR DRUG SUBSTANCE AND DRUG PRODUCT

Though many types of HPL chromatographic techniques are available, the most commonly submitted method, the reversed-phase HPLC with UV detection, is selected to illustrate the parameters for validation. The criteria for the validation of this technique can be extrapolated to other detection methods and chromatographic techniques. For acceptance, release or stability testing, accuracy should be optimized since the need to show deviation from the actual or true value is of the greatest concern.

A. Accuracy

Accuracy is the measure of how close the experimental value is to the true value.

Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guideline for Submitting Samples and Analytical Data for Methods Validation.

For the drug product, this is performed frequently by the addition of known amounts of drug by weight or volume (dissolved in diluent) to the placebo formulation working in the linear range of detection of the analyte. This would be a true recovery for liquid formulations. For formulations such as tablet, suppository, transdermal patch, this could mean evaluating potential interaction of the active drug with the excipients in the diluent. From a practical standpoint, it is difficult to manufacture a single unit with known amount of active drug to evaluate recovery. This test evaluates the specificity of the method in the presence of the excipients under the chromatographic conditions used for the analysis of the drug product. It will pick up recovery problems that could be encountered during the sample preparation and the chromatographic procedures. However, it does not count the effect of the manufacturing process.

At each recommended level studied, replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method is. The mean of the replicates, expressed as % label claim, indicates how accurate the test method is.

Recommendations:

Recovery data, at least in triplicate, at each level (80, 100 and 120% of label claim) is recommended. The mean is an estimate of accuracy and the RSD is an estimate of sample analysis precision.

B. Detection Limit and Quantitation Limit

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product.

Detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated

experimental conditions. Quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer. At low levels, assurance is needed that the detection and quantitation limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear/appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. For example, detection limit claim of 0.01% for the analyte integrated area count of 50,000 will give an area count of 5 that is not detectable.

Though USP expresses detection limit and quantitation limit in terms of 2 or 3, and 10 times noise level respectively, this concept is not very practical. Noise level on a detector during the method development phase may be different when samples are assayed on different detectors, etc. The use of standard(s) in the test method at the quantitation limit level (proposed by the applicant) is assurance that the impurity can be observed and quantitated.

Detector sensitivity can vary with the model number and/or manufacturer as illustrated in Table 1 for the analysis of a compound by two commercial detectors. The data should not be taken as the expected ratio of sensitivity of the two detectors. It is not known if other parameters which can also play a part, e.g., age of lamp, column, were considered when setting these limits.

Table 1. Comparison of Detector Sensitivity Limits in Two Commercial Detectors.

	Detector 1	Detector 2
Quantitation Limit	0.21%	0.07%
Detection Limit	0.16%	0.05%

One also should be cautious that baseline noise is not interpreted as extraneous peaks. Undulations may be observed at the void volume if the diluent for the sample is different from the solvents (proportion and type) used in the mobile phase.

If a reference standard for the compound of interest is available, a standard close to the quantitation limit or the specification could be used. For monitoring peak(s) with no reference standard for the impurity, a diluted reference standard of the drug substance is recommended. The method should then check that the high and low concentrations are operating in the linear range of detection of the drug substance. Otherwise the information that is expressed as % area or height of the drug substance peak from the same HPL chromatogram will be biased. It should also be noted that the extraneous peak using area count does not consider the detection response which depends on the UV extinction coefficient or absorptivity of the compound.

Recommendations:

1. Analysis repeatability and injection repeatability data at the quantitation limit.
2. Use of an additional reference standard at the quantitation limit level in the test method.

C. Linearity

The linear range of detectability that obeys Beer's Law is dependent on the compound analyzed and detector used. The working sample concentration and samples tested for accuracy should be in the linear range.

Figures 1 and 2 illustrate the behavior of UV response vs. concentration of a (a) linear and (b) non-linear relationship. A point to note is that when monitoring impurity peaks expressed as percent area of the parent drug substance, the impurity observed may not be a true reflection of the theoretical amount if the non-linear section of the concentration curve is employed. In addition, the actual amount will be obtained only if the extinction coefficient or absorptivity values are the same for both impurity and parent compound. Impurity reference standards are often needed.

Figure 1. Concentrations vs. Peak Areas of Standards to Illustrate Linearity.

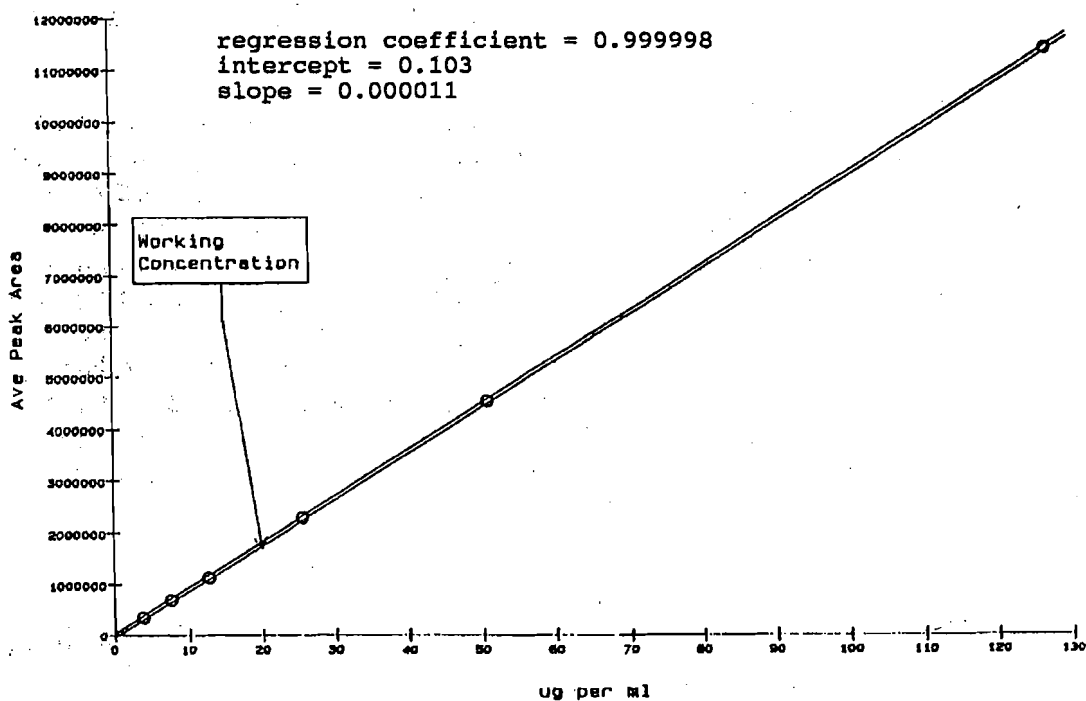
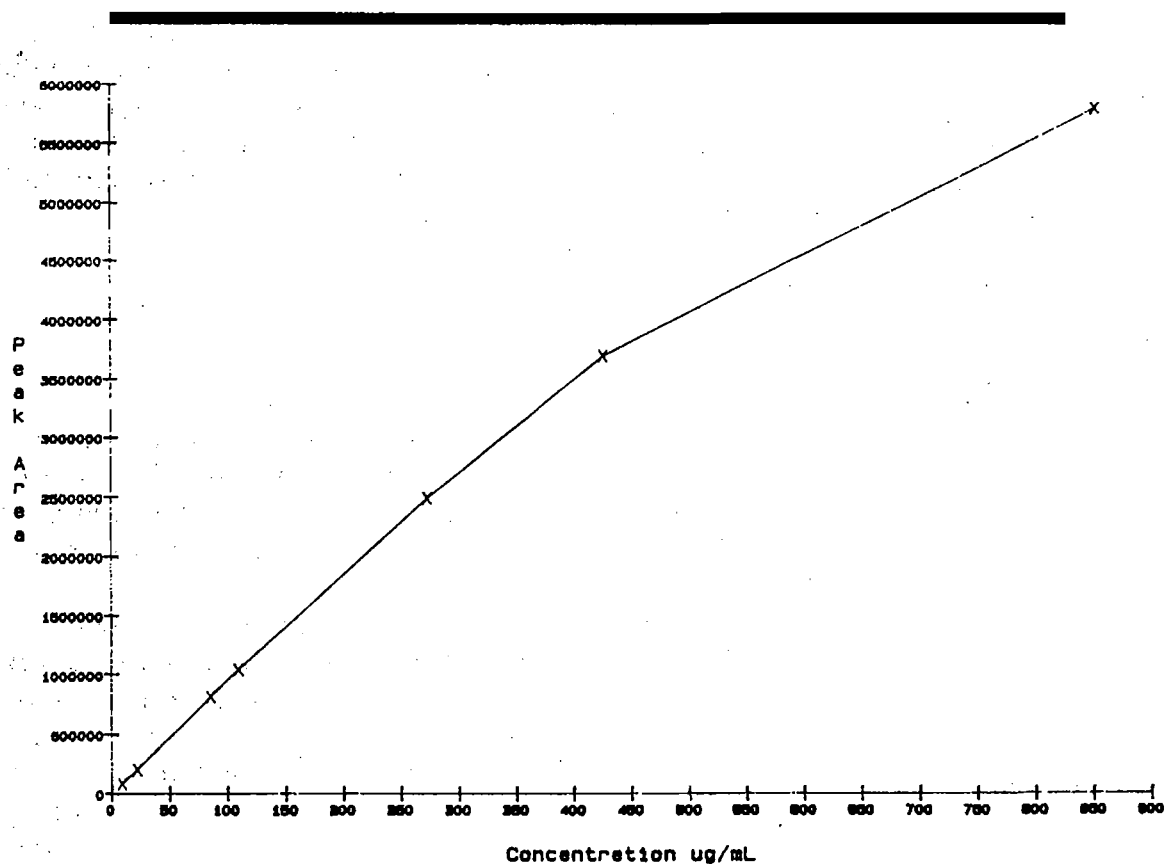


Figure 2. Concentrations vs. Peak Areas of Standards Outside the Linear Range.



Recommendations:

The linearity range for examination depends on the purpose of the test method. For example, the recommended range for an assay method for content would be $NLT \pm 20\%$ and the range for an assay/impurities combination method based on area % (for impurities) would be $+20\%$ of target concentration down to the limit of quantitation of the drug substance or impurity. Under most circumstances, regression coefficient (r) is ≥ 0.999 . Intercept and slope should be indicated.

D. Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in USP XXII <1225>, 1990 incorporates the concepts described under the terms "intermediate precision", "reproducibility" and "robustness" of this guide.

1. Repeatability

a. Injection Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing).

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested. The information is provided as part of the validation data and as a system suitability test. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. Keep in mind, however, that it does not consider variations due to the drug product manufacturing and laboratory sample preparation procedures. As an illustration for injection and R_t variation, Table 2 provides representative data collected when a leak developed in the chromatographic system during sampling. The set of four duplicate samples were injected sequentially. Variations in peak area and drift of retention times are noted. Sets of typical data from a well-behaved system for comparison are shown in Table 3.

Table 2. Representative Injection Repeatability Data for an HPL Chromatographic System that Developed a Leak During Sampling.

Sample	R _t	Peak Area	Δ R _t	Δ Peak Area
A1 A2	5.62 5.66	2155699 2120466	0.04	35233
B1 B2	5.87 6.13	2205659 2288355	0.26	82696
C1 C2	6.21 6.48	2227066 2265279	0.27	38213
D1 D2	6.73 6.99	2581888 2602016	0.26	20128

Table 3. Representative Injection Repeatability Data for Select Formulations from a Normally Functional HPL Chromatographic System.

Dosage Form	n	Mean ± SD	RSD
Inhalation Solution	10	1993162 ± 5029	0.25%
Solution for Inhalation	10	1722253 ± 6288	0.37%
Capsule	10	1744320 ± 3133	0.18%

Recommendations:

As part of methods validation, a minimum of 10 injections with an RSD of $\leq 1\%$ is recommended. With the methods for release and stability studies, an RSD of $\leq 1\%$ RSD for precision of the system suitability tests for at least five injections ($n \geq 5$) for the active drug either in drug substance or drug product is desirable. For low level impurities, higher variations may be acceptable.

b. Analysis Repeatability

Determination, expressed as the RSD, consists of multiple measurements of a sample by the same analyst under the same analytical conditions. For practical purpose, it is often combined with accuracy and carried out as a single study. See section IV.A under Accuracy.

2. Intermediate Precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over.

Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

Intermediate precision in the test method can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

Recommendations:

As a minimum, data generated as described under section IV.A Accuracy, for two separate occasions, is recommended to indicate the intermediate precision of the test method.

3. Reproducibility

As defined by ICH, reproducibility expresses the precision between laboratories as in collaborative studies. Multiple laboratories are desirable but not always attainable because of the size of the firm.

Recommendations:

It is not normally expected if intermediate precision is accomplished.

E. Range

Range is the interval between the high and low levels of analyte studied. See also sections IV.A and C under Accuracy and Linearity respectively.

The ranges recommended in sections IV.A and C under Accuracy and Linearity can be applied to other analytes, e.g., preservatives.

F. Recovery

Recovery is expressed as the amount/weight of the compound of interest analyzed as a percentage to the theoretical amount present in the medium.

Full recovery should be obtained for the compound(s) of interest. During the sample preparation procedure, the compound of interest is recovered from excipients in the formulation matrix ranging from a simple aqueous solution to complex cream formulation, and from potential adhesion to container/closure components, e.g., glass vial, metered valve. In general, a simpler sample preparation procedure will result in a lower variation of recovery. Data collection for recovery are discussed in section IV.A under Accuracy.

G. Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

Testing varying some or all conditions, e.g., age of columns, column type, column temperature, pH of buffer in mobile phase, reagents, is normally performed.

Recommendations:

Data obtained from studies for robustness, though not usually submitted, are recommended to be included as part of method validation.

H. Sample Solution Stability

Solution stability of the drug substance or drug product after preparation according to the test method should be evaluated according to the test method. Most laboratories utilize autosamplers with overnight runs and the sample will be in solution for hours in the laboratory environment before the test procedure is completed. This is of concern especially for drugs that can undergo degradation by hydrolysis, photolysis or adhesion to glassware.

Recommendations:

Data to support the sample solution stability under normal laboratory conditions for the duration of the test procedure, e.g., twenty-four hours, should be generated. In exceptional cases where multiple days are needed for sample preparation or solution storage, an appropriate stability time should be selected.

I. Specificity/selectivity

The analyte should have no interference from other extraneous components and be well resolved from them. A representative HPL chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte. Examples of the extraneous peaks are as follows:

- For the drug substance or raw material, the related substances to consider are process impurities (which include isomeric impurities) from the synthesis process, residual pesticides, solvents, and other extraneous components from extracts of natural origin.

- For the drug product, the related substances may be impurities present in the active drug, degradation products, interaction of the active drug with excipients, extraneous components, e.g., residual solvents from the excipients or manufacturing process, leachables or extractables from the container and closure system or from the manufacturing process.

Submission of data from stress testing of the drug substance using acid and base hydrolysis, temperature, photolysis and oxidation according to the Guideline for Submitting Samples and Analytical Data for Methods Validation is recommended. Representative HPL chromatograms are recommended for stressed and non-stressed samples that include test methods for impurities, preservatives, etc. and placebo sample. With the impurities test method, the HPL chromatogram should indicate the presence of impurities at the level of detection/quantitation claimed. The chromatograms should be legible, labeled, and the time or time scale and attenuation should be indicated.

Points to note are as follows:

1. The parent peak may be expanded, e.g., by increasing the concentration, attenuation change, so that extraneous peaks can be observed at a reasonable size to evaluate stability-indicating capability. See comments in section IV.B under Limits of Detection/Quantitation.
2. The baseline should be on-scale as off-scale baseline (observed as a flat straight line) can hide minor peaks.

Peak purity can be determined by the photo-diode array detector. Low level extraneous components present under the compound of interest, however, may not interfere or influence the UV spectrum of the analyte.

Figures 3 and 4 illustrate the combination of UV spectroscopy and HPL chromatography by photo-diode array detection using (a) 3-dimensional plot and (b) conventional HPL chromatogram. The analyte elutes at 4.7 minutes. It should be noted that the quality of the UV spectra for the low level components is poor.

Figure 3. A Representative 3-Dimensional Plot of the HPL Chromatogram with the UV Spectra.

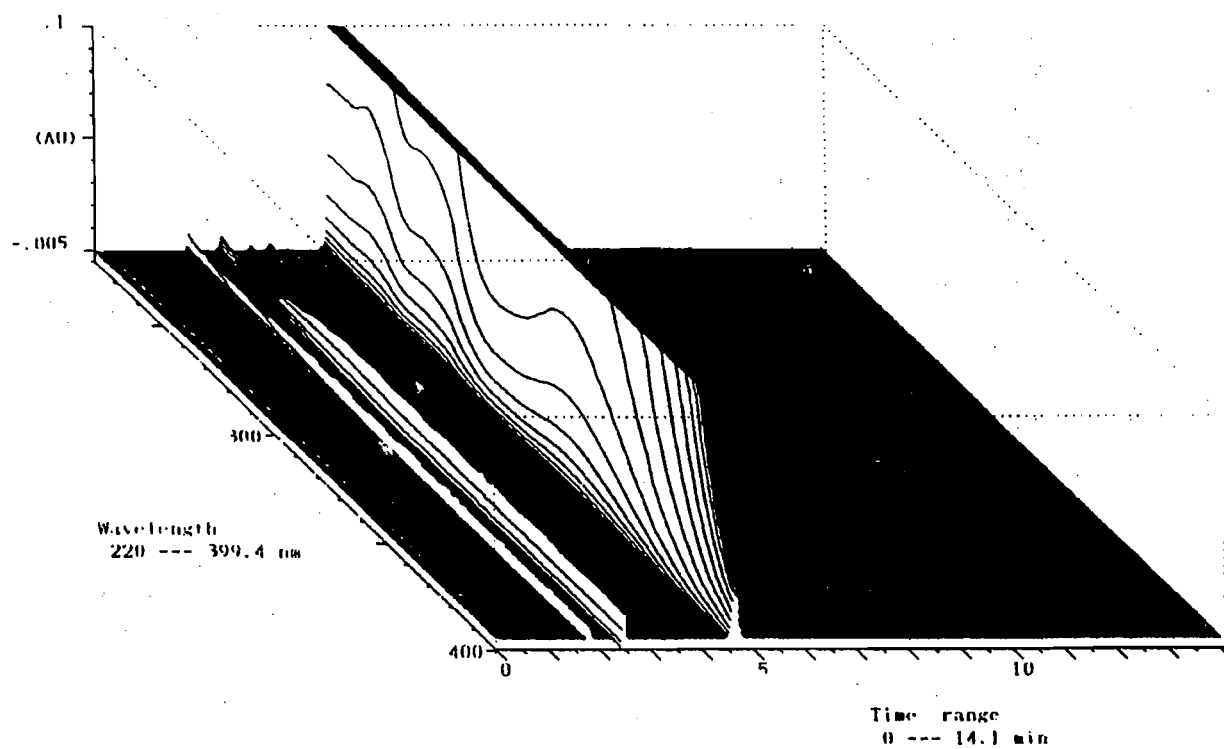
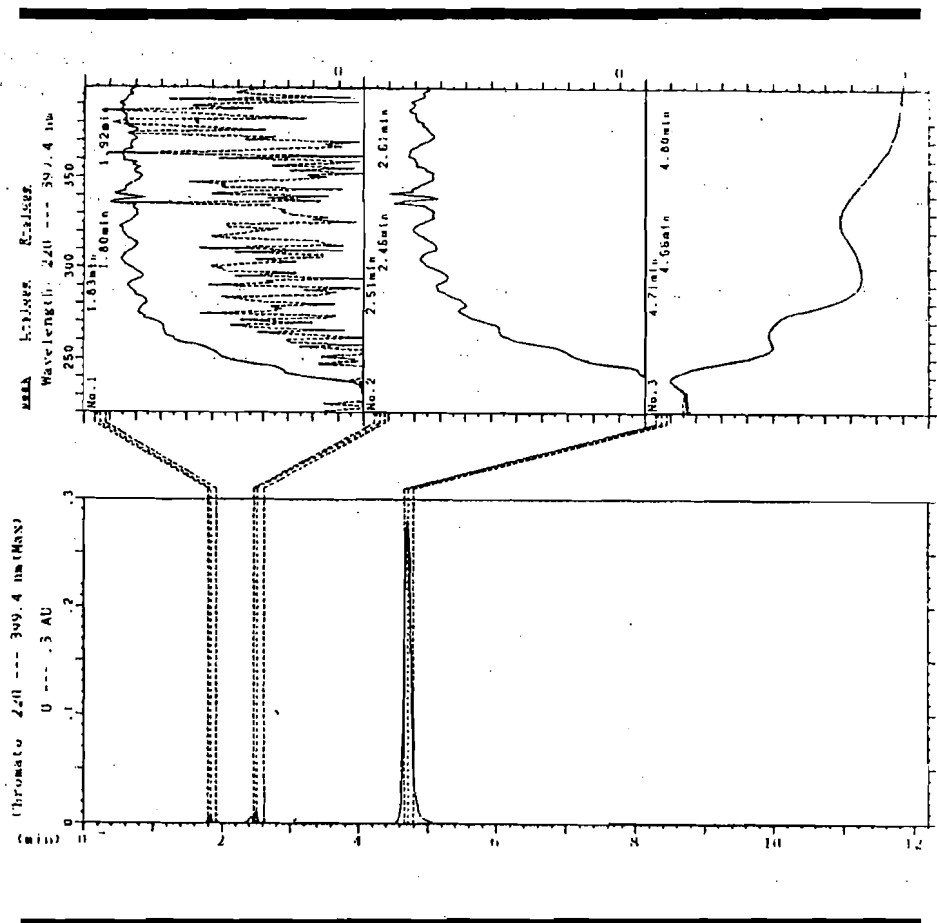


Figure 4. A Representative Conventional HPL Chromatogram with UV Spectra.



When stressed samples are used, an appropriate detector/integrator setting should be selected. For example, to be able to detect low levels, e.g., 0.1% degradation products, the parent peak should be of a size that at least a 0.1% detectability or area count is feasible.

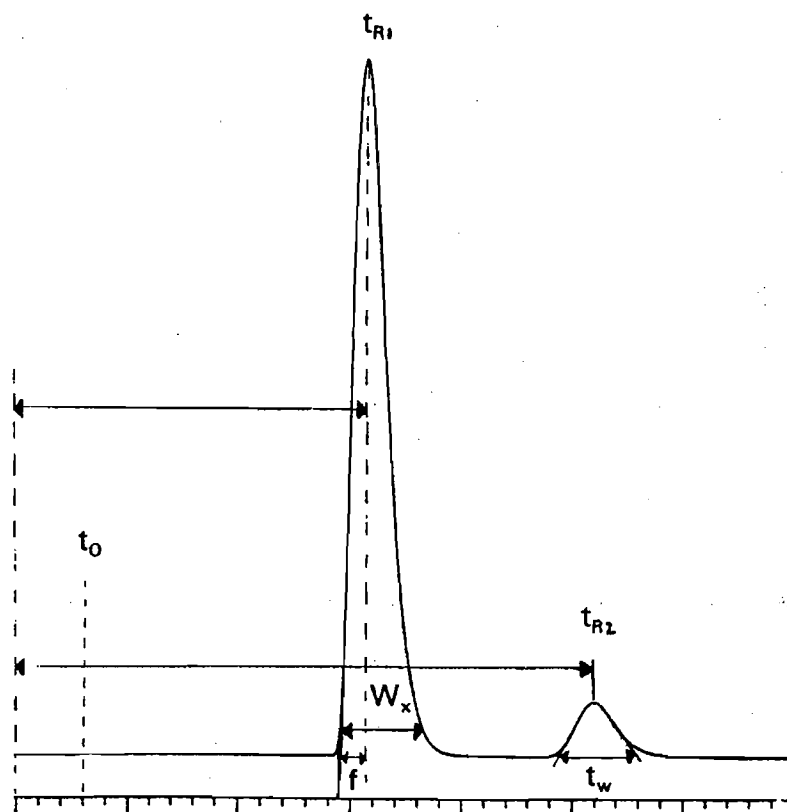
Recommendations:

Representative HPL chromatograms should be submitted for stressed and non-stressed samples that include impurities test method, preservative(s), etc. with the related placebo sample. Representative HPL chromatogram(s) to show selectivity by the addition of known extraneous compounds also should be submitted.

J. System Suitability Specifications and Tests

The accuracy and precision of HPLC data collected begin with a well-behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose. This section explains the terms as indicated in Figure 5, and provides recommendations and illustrations.

Figure 5. Definition of Terms for the System Suitability Parameters.



Where

- W_x = width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height
- f = distance between peak maximum and peak front at W_x
- t_0 = elution time of the void volume or non-retained components
- t_R = retention time of the analyte
- t_w = peak width measured at baseline of the extrapolated straight sides to baseline

t_R = retention time of the analyte
 t_W = peak width measured at baseline of the extrapolated straight sides to baseline

1. Capacity factor (k')

$$k' = (t_R - t_0) / t_0$$

The capacity factor is a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non-retained components.

Recommendations:

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2 .

2. Precision/Injection repeatability (RSD)

Injection precision expressed as RSD (relative standard deviation) indicates the performance of the HPL chromatograph which includes the plumbing, column, and environmental conditions, at the time the samples are analyzed. It should be noted that sample preparation and manufacturing variations are not considered.

Recommendations:

RSD of $\leq 1\%$ for $n \geq 5$ is desirable.

3. Relative retention (α)

$$\alpha = k'_1 / k'_2$$

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (R_s) is stated.

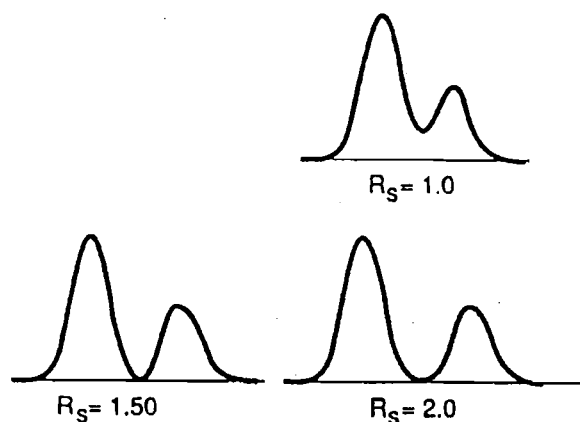
4. Resolution (R_s)

$$R_s = (t_{R2} - t_{R1}) / (1/2) (t_{W1} + t_{W2})$$

R_s is a measure of how well two peaks are separated. For reliable quantitation, well-separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak(s) may be of concern. The closest potential eluting peak to the

R_s is minimally influenced by the ratio of the two compounds being measured. The resolution of peaks as indicated by the R_s values is shown in Figure 6.

Figure 6. Separation of Peaks as Indicated by R_s Values.



Recommendations:

R_s of > 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) is desirable.

5. Tailing factor (T)

$$T = W_x / 2f$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the

peak of interest. Figures 7 and 8 illustrate the tailing factors and the effect on quantitation. If the integrator is unable to determine exactly when an upslope or downslope occurs, accuracy drops.

Figure 7. HPLC Peak with Various Tailing Factors.

Tailing factor = 1.3

Tailing factor = 3.7

Tailing factor = 4.4

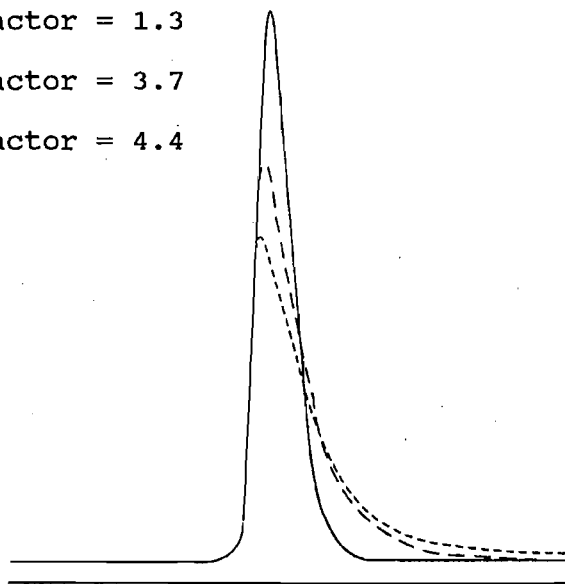
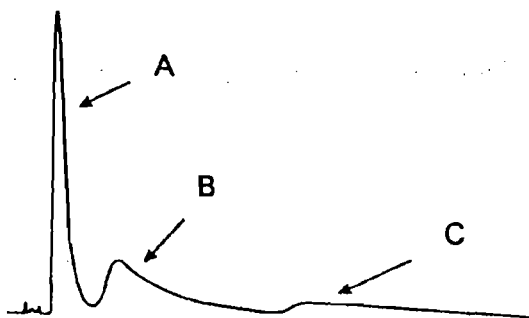
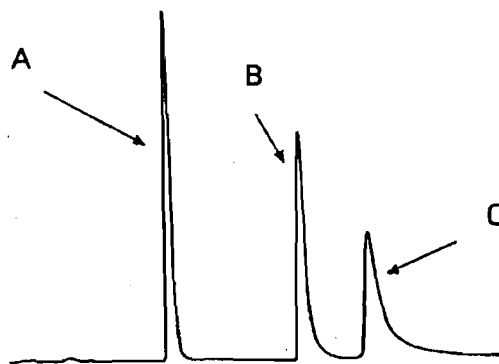


Figure 8. Effect of Peak Tailing on Quantitation.



Recommendations:

T of ≤ 2

6. Theoretical plate number (N)

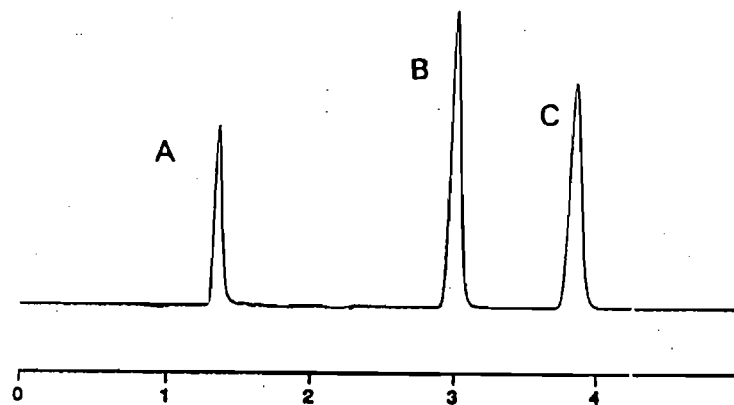
$$N = 16 (t_R / t_W)^2 = L / H$$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram.

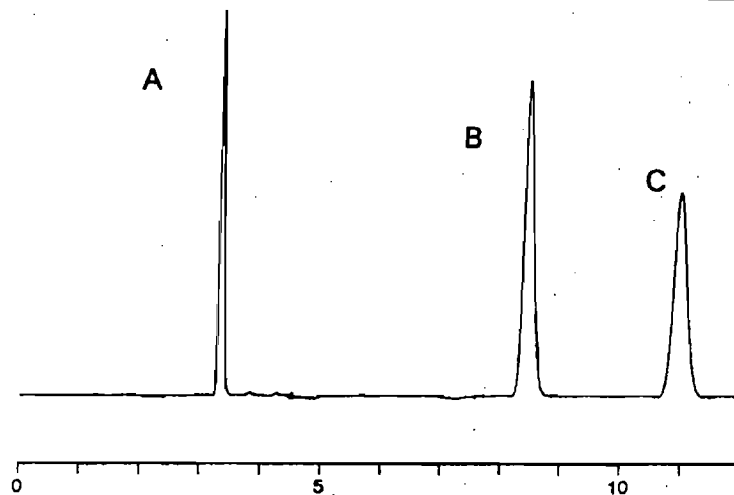
N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H, or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte. Figure 9 shows one set of compounds A, B, and C under two different chromatographic conditions resulting in, e.g., R_t s for B to be 3 and 8.5 minutes, respectively. An examination of peak B indicates that the theoretical plate values are different even though the peaks appear similar visually.

Figure 9. Effect of Retention Times on Theoretical Plates.

	R_t	N	k'
A	1.35	2007	0.51
B	3.00	4702	2.35
C	3.85	5929	3.29



	R_t	N	k'
A	3.36	5076	0.60
B	8.46	7175	3.03
C	10.99	8742	4.23



Recommendations:

The theoretical plate number depends on elution time but in general should be > 2000 .

General Recommendation:

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, k' , T and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, k' , T , R_s and RSD are recommended as minimum system suitability testing parameters. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system. Additional tests may be selected at the discretion of the applicant or the reviewer.

K. General Points to Consider

Some basic points to note in the test method are:

1. The sample and standard should be dissolved in the mobile phase. If that is not possible, then avoid using too high a level of the organic solvent as compared to the level in the mobile phase.
2. The sample and standard concentrations should be close if not the same.
3. The samples should be bracketed by standards during the analytical procedure.
4. Filtration of the samples before injection is occasionally observed. Filtration will remove particulates (centrifugation performs the same function) that may clog columns. Adhesion of the analyte to the filter can also happen. This will be of importance especially for low level impurities. Data to validate this aspect should be submitted by the applicant.

V. COMMENTS AND CONCLUSIONS

HPL Chromatographic Methods for Drug Substance and Drug Product.

Methods should not be validated as a one-time situation, but methods should be validated and designed by the developer or user to ensure ruggedness or robustness throughout the life of the method.

The variations due to the drug product manufacturing process, the laboratory sample preparation procedure and the instrument performance contribute to the accuracy of the data obtained from the analysis. With proper validation and tight chromatographic performance (system suitability) criteria, an improvement in the reliability of the data can be obtained. Variations, except from the drug product manufacturing process, will be minimized. Only with good reliable validated methods, can data that are generated for release, stability, pharmacokinetics be trust-worthy.

VI. ACKNOWLEDGEMENTS

Comments from Drs. Hoiberg, Poochikian, Blumenstein, Schroeder, Look, Tolgyesi (HFD-150); Dr. Layoff (HFH-300); Drs. Zimmerman and Piechocki (HFD-110); and the Analytical Methods Technical Committee: Dr. Sheinin, Mr. Shostak, Ms. Cunningham, Ms. Jongedyk, Mr. Leutzinger, Dr. Seggel, Ms. Sharkey and Mr. Smela are appreciated.

VII. REFERENCES

1. Guideline for Submitting Samples and Analytical Data for Methods Validation, February 1987.
2. United States Pharmacopeia, XXII, 1990. <1225>.
3. Text on Validation of Analytical Procedures, International Conference on Harmonization, September 1993.

Submitted by:

Linda L. Ng, Ph.D.

Approved by CMC CC:

Charles Kumkumian, Ph.D.

Roger L. Williams, M.D.