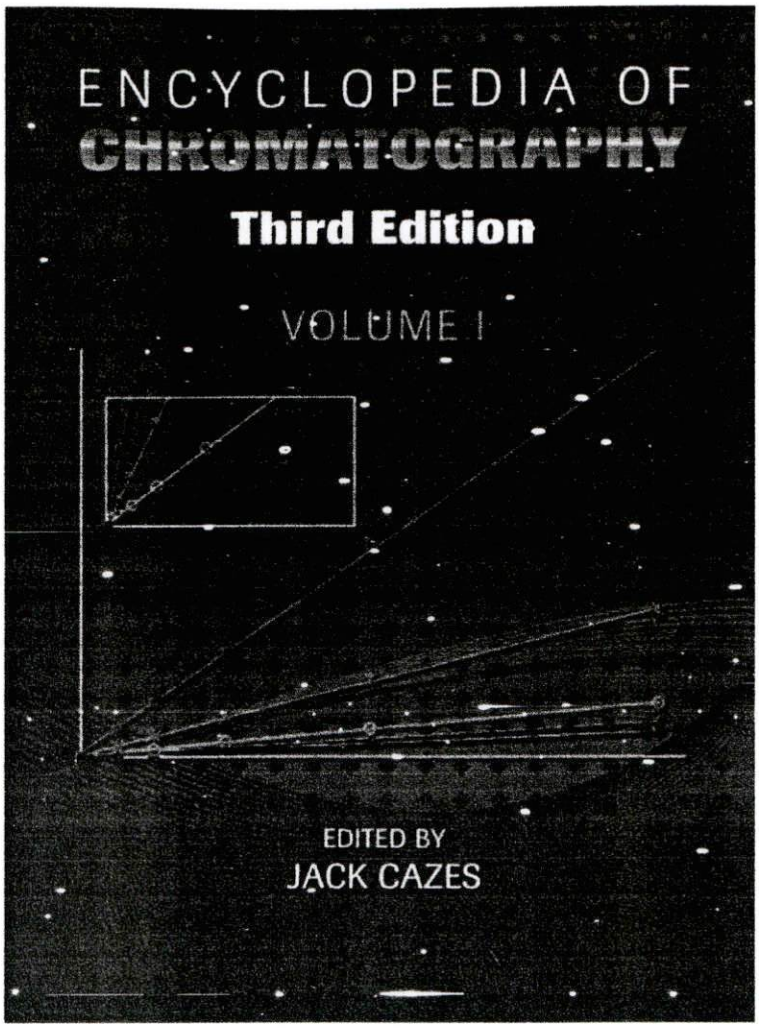
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TLC: Validation of Analyses

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TLC: Validation of Analyses

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Abstract
For obtaining reliable analysis results, the (high-performance) thin-layer chromatographic (TLC) method should be validated before using it as a quality control tool. The validation parameters that should be evaluated are stability of the analyte, specificity/selectivity, linearity, accuracy, precision, range, detection limit, quantification limit, and robustness/ruggedness.

INTRODUCTION

According to USP 30–NF 25,[1] validation of an analytical method is the process by which it is established, by laboratories studies, that the performance characteristics of the method meet the requirements for the intended analytical applications. Therefore, validation is an important step in determining the reliability and reproducibility of the method because it could confirm that the method is suitable to be conducted on a particular system. The performance parameters that should be determined in validation studies include specificity/selectivity, linearity, accuracy, precision, detection limit (DL), quantitation limit (QL), range, ruggedness, and robustness.[1,2] The parameters that are required to be validated depend on the type of the analyses; thus, different test methods require different validation schemes. The most common types of analyses are identification, quantitative determination of impurities, limit value determination of impurities, and quantitative determination of active ingredients.[3] Besides the general validation parameters mentioned above, the software and hardware of the thin-layer chromatographic (TLC) scanner should be validated first.[3,4] The validation of the instrument is categorized into design qualification (DQ), installation qualification (IQ), operation qualification (OQ), and performance qualification (PQ).[3]

STABILITY TESTING

A very important prevalidation method requirement is to test the stability of standards and samples. The analyte should be stable during sample preparations (at least 30 min) on the sorbent surface before development (at least 30 min) and during development (at least 1 hr).[5] The acceptance criteria are within 2%. The age mobile phase should also give the same value of R_f, T (tailing factor), and N (theoretical plate). The stability of the analyte should also be tested in biological matrices during storage. Reconstituted samples must remain stable in the solvents at the working temperature until spotted.[6] For fingerprint of herbal drugs, Reich, Schibli, and DeBatt[7] recommended to test the stability of analytes in solution and on the plate over 3 hr. Stability during chromatography should be tested using 2-dimensional development. If visualization requires a derivatization step, the stability must also be evaluated. Reich, Schibli, and DeBatt[7] suggested taking one image immediately after 5, 10, 20, 30 min, and 1 hr of derivatization, then the images are compared visually and by using video densitometry.

SPECIFICITY/SELECTIVITY

The terms specificity and selectivity are often used interchangeably. A method is said to be specific if it provides a response for only a single analyte. If the response in question is distinguished from all other responses, the method is said to be selective.[8] The International Conference on Harmonization (ICH)-2 does not differentiate both terms and defines specificity or selectivity as "the ability to unambiguously determine the analyte in the presence of other components whose presence is to be expected." This includes typical impurities, decomposition products, and matrix components.[3] The specificity of the method for TLC or high-performance TLC (HPTLC) analyses was proved by identification and purity checks of the analyte spots. This can be done by measuring in situ the ultraviolet and visible spectroscopy (UV–Vis) spectra of the analyte(s) and the authentic reference standard(s), those eluted on the same plate, and then by calculating their correlations (the r value should be ≥ 0.999). This correlation should be calculated on the upslope, the apex, and the downslope of the peaks. In a quality control laboratory, the selectivity

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can be proven easily by spotting the standard, blank sample, possible impurities, or degradation products in the proposed system, analyte spot must not be interfered by other spots, which can be proven by calculating the resolution. If sources for impurities and degradation products are not available, the samples should be exposed to stress condition such as heat, UV light, acid, and base.[6] Other parameters should ideally be in the following range: R_f ($0.1 \leq R_f \leq 0.9$); tailing factor, T ($0.9 \leq T \leq 1.1$); resolution, R_s (≥ 1.0).[9] For proving the selectivity for analysis of botanical drugs, the availability of botanical reference material (BRM) is essential. It would be very nice if at least three authenticated BRM of each species from different origins can be used. This can be used to illustrate natural variability. A method is specific if during validation a sample representing target species showed identical fingerprint to that of BRM and samples representing other species give different fingerprints.[7]

LINEARITY

The linearity of a method is its ability to provide measurement results that are directly proportional to the concentration of the analyte or are directly proportional after mathematical transformation. The linearity is usually documented as the linear regression curve of the measured responses as a function of increasing analyte concentrations.[3] To perform linearity testing, it is recommended to use 5–10 concentrations with range equivalent to 80–120%,[9] 25–200%,[10] or 50–150%[11] of the expected content of the analyte. For the determination of degradant and preservative in the stability study, ranges of 0–40% and 50–110%, respectively, were recommended.[2] For dissolution testing ±20% over the specified concentration is tested, while for content uniformity a minimum of 70–130% of concentration is tested.[1] It is also essential that the basic calibration be performed by using independent samples and not by using samples that have been prepared by dilution and spotted on one TLC or HPTLC plate. For the evaluation of basic calibration line, several parameters can be used, for example, correlation coefficient (r), the relative process standard deviation value (V_{xo}), the Mandel's test, the X_p value,[12] the plot of response factor vs. concentration, the residual test, the analysis of variance (ANOVA), and so on. The lowest concentration used for the calibration curve must not be less than the value of X_p.[12] Camag (Muttents) calculated by using its CATS software the value of *sdv* [relative standard deviation (RSD) of the calibration curve] for expressing the linearity. If the reader is working with CATS software, the smallest value of *sdv* should be selected to determine the most suitable regression model that will be used for calibration. It is recommended that the correlation coefficient (r) alone not be used anymore. The correlation coefficient does not indicate the linearity or the lack thereof.[13] Readers should refer to previous works for further discussion.[12,14] The variance homogeneity over the whole range of the basic calibration line should also be proved using the F test.[9,12] If working range (WR) is defined as the ratio of the highest and lowest concentration of standards of the calibration or linear curve, it is recommended that a WR of not more than 10 be used; if WR is more than 10, a weighting regression should be used.[15] For quantitative analysis using TLC or HPTLC, the calibration curve must be performed on each plate; each plate should contain standards and unknown samples.

ACCURACY

Accuracy or trueness of an analytical method is given by the extent to which the value obtained deviates from the true value.[3] In the first approach, accuracy can be determined by analyzing a sample with known concentration and then by comparing the results between the measured and the true value. The second approach is by comparing the test results obtained from the new method to the results from the existing method that is known to be accurate. The third and fourth approaches are based on the percent recovery of known analyte spiked into blank matrices or products. The last technique is known as the standard addition method.[11] For spiking analyte into blank matrices, it is recommended to prepare the sample (laboratory-made preparation) in five different concentrations at the level of 80–120% of the target concentration. Experiences from our laboratory have shown that by using at least five levels of concentrations in duplicate (i.e., 80%, 90%, 100%, 110%, and 120% of target concentration), an accurate result can be achieved. On the contrary, for the standard addition method, the spiking concentrations are 30–60% of the label-claimed value. For dissolution studies, the accuracy should be tested at 40%, 75%, and 110% of the theoretical release.[6] To prove whether systematic errors do not occur, a linear regression of recovery curve of X_f (concentration of the analyte measured by the propose method) against X_c (nominal concentration of the analyte) should be constructed, and the confidence range of the intercept {$VB(a_f)$} and slope {$VB(b_f)$} from the recovery curves should be calculated for $p = 0.05$.[12]

PRECISION

The realistic standard deviations in TLC or HPTLC analyses are ~0.2% on multiple scanning of one spot, 0.8–1.5% on multiple spotting and analysis of the same sample solution, and 1.5–2.0% on multiple analysis of the same sample.[9] As a general rule, the standard deviation of a method should be lower than 1/6 of the specification range,[16] or the RSD value should not be more than 2%.[10] For validation purposes, precision is determined

by muliple application of the complete analytical procedure on one homogenous real sample. According to ICH, both repeatability and intermediate precision should be tested.[6] Repeatability is defined as precision under the same conditions, that is, same analyst, equipment, reagents, time, and TLC plate. Intermediate precision was performed by repeatability testing on the different combinations of analyst, equipment, reagents, and time within one laboratory. It is recommended to do 6–10 measurements on each repeatability studies.[9,10] A detailed discussion on the maximum of standard deviation for assay determination was published.[16] Other parameters that should be tested in the precision study are the David, Dixon or Grubbs, and Neumann test. Detailed discussion has been provided by Kromidas.[17] Reich, Schibli, and DeBatt[7] reported a detailed method to evaluate precision for qualitative analysis, in this case for HPTLC fingerprint of herbal drugs. They recommended to use at least three portions of the BRM and spotted onto three different plates. The fingerprint must be identical; the variability of R_f values of three markers should not exceed 0.01 across each plate, 0.02 for repeatability, 0.05 for intermediate, and 0.07 for reproducibility.

RANGE

The range of measurement is the interval for which linearity, accuracy, and precision have been tested. Analysis outside the range is not permitted.[1,3] For quality control purposes in a pharmaceutical industry, it is recommended to use a range from 80% to 120% of the target concentration, and for dissolution ±20% from upper and lower limit, respectively. For pharmacokinetic study, a wide range should be tested, the maximum value should exceed the highest expected concentration, and the minimum value is the QL.

DETECTION LIMIT AND QUANTITATION LIMIT

DL is defined as the lowest concentration of an analyte that can be detected under the analytical condition to be used. However, it cannot be quantitatively measured. QL is the lowest concentration that can be determined with acceptable accuracy and precision under the analytical conditions.[3] Generally, QL can be estimated as three times of DL.[2,14] DL and QL for instrumental (chromatographic) analytical methods can be defined in terms of the signal-to-noise ratio (2:1 to 3:1 for DL and 10:1 for QL[9] or in terms of the ratio of the standard deviation of blank response, the residual standard deviation of the calibration line, or the standard deviation of intercept (σ) and slope (S) (DL = $3.3\sigma/S$ and QL = $10\sigma/S$).[16] By constructing a linear regression of relatively low concentrations of analyte, accurate values of DL can be calculated, in this case, DL = X_p.[12] The authors recommend using 5–10 relatively low concentrations of analyte spotted on a TLC plate. DL can be increased by using pre- or postchromatographic derivatization.[3] For clinical application, QL should be at least 10% of the minimum effective concentration, and it should be within the linear WR.[6] For ensuring the safety of agricultural products, herbal medicines, and foods from the contamination of certain heavy metals, antibiotics, pesticides, and drugs by chemical analysis, DL of the method must be determined. DL should be less than the maximum permitted level of the toxic substances.

ROBUSTNESS/RUGGEDNESS

Robustness can be defined as a measure of the capability of the method to remain unaffected by small, but deliberate, variations in method parameters. It provides an indication of its reliability during normal usage.[1,18] Ruggedness of a method is the degree of reproducibility of test results obtained by the analysis of same samples under a variety of conditions such as different laboratories, analysts, and instruments; different lot of reagents; different days.[1] Some important parameters for the testing of TLC or HPTLC methods are the following: the stability of analyte in the solution being analyzed and on the plate before and after development; the influence of temperature and humidity; the method of application, scanning, and evaluation; the spot shape and size, eluent composition, and pH; the batch and sources of TLC plate; the sample volume; different chamber type; and the drying conditions of the plate.[3,18] In our laboratory, the design and analysis of effect of the robustness data were performed and calculated by using a multivariate statistical software. As an example, we studied the effect of small variation in the composition of mobile phase on R_f, T, and recovery of the active ingredient.[19] A detailed guidance for robustness/ruggedness testing has been published.[16] For robustness evaluation of botanical drugs, the R_f values of all markers should lie within the acceptance criteria of the intermediate precision.[7]

REPORTING RESULTS OF ANALYSIS

For reporting routine analytical results, it is recommended that its confidence interval[12] or uncertainty[17] or standard certainty[20] be included in the results of analysis; so the result is reported as:

$$\text{Mean} \pm \frac{t.\text{SD}}{\sqrt{N}}$$

where N is the number of replicates, SD is the standard deviation, and t is the value from the t table; if N is more than 10, the equation can be simplified as:[17]

Mean $\pm$ 2 $\times$ SD (for $p = 0.05$)

Mean $\pm$ 3 $\times$ SD (for $p = 0.01$)

Kaiser[20] suggested four replicates for analyzing samples, and in this case standard certainty can be estimated as three times the standard deviation.

CONCLUDING REMARKS

Important performance characteristics for TLC or HPTLC validation procedures have been described in brief in this entry to provide guidance to the analytical chemists. For obtaining reliable and reproducible analysis results, it is essential to validate the TLC or HPTLC method first, before using it for routine works in a quality control laboratory.

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