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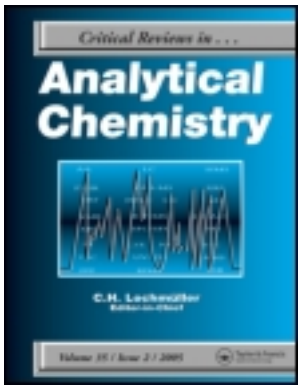

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Analytical Validation of Quantitative High-Performance Liquid Chromatographic Methods in Pharmaceutical Analysis: A Practical Approach

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Analytical Validation of Quantitative High-Performance Liquid Chromatographic Methods in Pharmaceutical Analysis: A Practical Approach

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Analytical validation is an essential component in allowing a laboratory to ensure routine acceptable performance of analytical methods. Despite the considerable amount of important published work on this subject, diversity still prevails in the employed methodologies because validation of an analytical method depends on the specific purpose of that method. This can lead to difficulties in validation approaches and the interpretation of results. Aiming to assist in the planning of validation methods, we discuss relevant approaches of various parameters in quantitative high-performance liquid chromatographic methods and validation fields in pharmaceutical analysis. Moreover, this article provides several up-to-date examples that should be useful as an introduction to analytical validation for practical applications in academic research or the industrial sector.

Keywords analytical method validation, quality control, high-performance liquid chromatographic methods, pharmaceutical analysis

INTRODUCTION

Validation is defined as "confirmation through examination and provision of objective evidence that the requirements for a specified intended use or application are fulfilled" (International Organization for Standardization, 2005). The execution of analytical validation procedures, therefore, supplies the scientific demonstration that an analytical method is suitable for its specific application. This approach is important in the circumstances of new method development, established method revision, the same method used in different laboratories, the employment of different analysts or instrumentation, and for demonstration of equivalence between two different methods (Eurachem Guide, 1998). These considerations lead us to conclude that method validation is an essential component in allowing a laboratory to ensure routine acceptable performance of analytical methods.

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Method validation has been published extensively in the literature, as well as in several guidelines from regulatory agencies, including Eurachem (Eurachem Guide, 1998), the International Union of Pure and Applied Chemistry (IUPAC; Thompson et al., 2000), AOAC (AOAC International, 2000), the U.S. Food and Drug Administration (FDA; FDA, 2000), the *United States Pharmacopoeia* (USP; *United States Pharmacopoeia*, 2009), International Conference on Harmonization (ICH; ICH, 2005), and World Health Organization (WHO; WHO, 1992). These guidelines serve as a worldwide basis both for regulatory authorities and industry.

The International Conference on the Harmonization of the Technical Requirements for Human Use Pharmaceuticals Registration (ICH) is a joint initiative involving the regulatory authorities of Europe, the U.S., and Japan. The ICH has an important and beneficial role in regulatory affairs in these three participating regions, as well as in many other regions throughout the world.

Despite the considerable importance of guidelines from the ICH and other study organizations, and the large amount of work published on analytical validation, a wide variety of validation methods still continue to be employed. The published guidelines disagree, however, on a few key points in validation

parameters and acceptance criteria (Chandran and Singh, 2007). These differences appear principally because the adopted procedures depend on the type and nature of the analytical method being performed, which can lead to difficulties in validation approaches and results interpretation. Moreover, analytical method validation requires an appreciation of the choice of performance characteristics, in addition to an understanding of statistical analysis.

In this article, we present a practical view of the concepts of various parameters in the validation fields of quantitative high-performance liquid chromatography (HPLC) analytical methods in pharmaceutical analysis, as well as several recent examples to serve as an introduction to applications of analytical validation, whether they are for an academic or an industrial researcher.

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ANALYTICAL METHOD VALIDATION PROCEDURES

According to ICH, there are four common types of analytical procedures to be validated: identification tests, impurities content quantitative tests, limit tests for impurity control, and quantitative tests for the active moiety or other selected component(s) in drug products (ICH, 2005). Once the analytical procedure type has been chosen, the laboratory will be able to define which parameters are appropriate for method validation. Table 1 presents characteristics that are normally evaluated for each analytical procedure type.

As observed in Table 1, in active moiety quantitative tests it is not necessary to perform detection limit (DL) and quantitation limit (QL) determinations. However, recent studies in the field of analytical validation for pharmaceutical analysis have determined DL and QL for these tests (Bonfilio et al., 2009; Fu et al., 2010; Jain et al., 2009; Kumar et al., 2009a; Mahadik et al., 2009; Rane et al., 2009; Shou et al., 2009). For this reason, this article considers these analytical validation parameters, and the authors recommend DL and QL determination to assure that the analytical validation is complete. Furthermore, determination of the DL and QL will demonstrate that the analyses are being performed in a region higher than these values, producing more reliable results.

The official guidelines do not present a validation experiment sequence because the optimal sequence may depend on the method itself. For quantitative analytical HPLC methods in pharmaceutical analysis, however, the following sequence has proven to be useful:

1. System suitability
2. Solution stability
3. Selectivity
4. Linearity and range
5. Precision (repeatability and intermediate precision)
6. Concentration range accuracy
7. Detection limit
8. Quantitation limit
9. Ruggedness

System Suitability

USP 31 guidelines (*United States Pharmacopoeia*, 2009) recommend the system suitability test as a component of any analytical procedure because HPLC data accuracy and precision begin with a well-behaved chromatographic system. This parameter is based on the concept that the samples, analytical procedures, electronics, and equipment to be checked constitute an integral system that can be evaluated as such (Shabir, 2003). The solution used must contain the analyzed compound at 100% of the level of that used in the method, including all other substances that could also be present in the sample (additional active ingredients, impurities, decomposition products, and internal standards) (Épshtein, 2004). The U.S. FDA

TABLE 1
Characteristics evaluated for each analytical procedures type (ICH, 2005)

Characteristics	Identification	Impurities Quantitation test	Impurities Limit test	Active moiety quantitative tests
Accuracy	−	+	−	+
Precision				
Repeatability	−	+	−	+
Intermediate Precision	−	+	−	+
Specificity	+	+	+	+
Detection Limit	−	−	+	−
Quantitation Limit	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

− This characteristic is not normally evaluated.
+ This characteristic is normally evaluated

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guidelines for pharmaceutical methods validation (U.S. Food and Drug Administration, 2000) describe the terms used in the system suitability test, as well as the acceptance criteria, that are useful for chromatographic system assessment. A brief description of these terms and their acceptance criteria are given in the following sections.

The capacity factor (K) is the retention time measurement of the peak of interest in relation to the nonretained component(s). This factor is expressed according to:

$$K = (t_R - t_o)/t_o \quad [1]$$

where t_R is the analyte retention time and t_o is the nonretained compound retention time. The FDA states that the analyte peak should be well resolved from the void volume with a K value > 2 (U.S. Food and Drug Administration, 2000).

Injection precision, expressed as an RSD (relative standard deviation), is determined through five or six injections performed in repeatability conditions of the same solution at 100% of the concentration used in the method. The RSD for injection precision is calculated by:

$$RSD(\%) = \frac{100SD}{\overline{X}} \quad [2]$$

where SD is the standard deviation determined in a series of measurements and $\overline{X}$ represents the mean value of the measurements. The FDA recommends that the RSD for injection precision be $\leq 1\%$ for n ≥ 5 (U.S. Food and Drug Administration, 2000).

Resolution (Rs) is a measure of how well two peaks are separated, and it is determined according to:

$$Rs = (t_{R2} - t_{R1})\big/(1/2)(w1 + w2) \quad [3]$$

where t_R is the retention time of the analyte and w is the peak width measured at the baseline of the extrapolated straight sides to baseline. Epshtein and Emshanova (2008) recommend $Rs \geq 1.5$, if the peaks have about the same height and a shape close to Gaussian. If the peaks have tailing or fronting, an $Rs \geq 2.0$ or even $Rs \geq 2.5$ is recommended.

The tailing factor (T), a peak symmetry measurement, is an important term in chromatographic analysis because the quantitation accuracy decreases with increases in the peak tailing. This effect is due to the difficulties encountered by the integrator in determining the area under the peak. T is obtained according to:

$$T = W_X\big/2f \quad [4]$$

where W_x is the peak width determined at either 5% (0.05) or 10% (0.10) above the peak baseline and f is the distance between the maximum of the peak and the front of the peak. As for the tailing factor, the FDA states that T should be ≤ 2 (U.S. Food and Drug Administration, 2000).

Theoretical plate number (N) is a measure of column efficiency and is constant for each peak on a chromatogram with fixed operating conditions. N is calculated from:

$$N = 16(t_R\big/t_W)^2 \quad [5]$$

where t_R is the analyte retention time and t_w is the peak width measured at the baseline of the extrapolated straight sides to the baseline. The theoretical plate number depends on elution time, but in general should be > 2000, according to the FDA (U.S. Food and Drug Administration, 2000).

A relevant example of analytical validation of an HPLC method in pharmaceutical analysis is the work of Karakuş and coworkers (Karakuş et al., 2008). The authors developed and validated a reversed-phase HPLC method for the determination of binary mixtures of pseudoephedrine hydrochloride (PSE) with fexofenadine hydrochloride (FEX) or cetirizine dihydrochloride (CET) in antihistaminic-decongestant pharmaceutical dosage forms. In this work, system suitability tests were determined by making six replicate injections from freshly prepared standard solutions in mobile phase. The authors analyzed theoretical plates (N), resolution (RS), and tailing factors (T) for each compound peak area. The acceptance criteria were an RSD for peak areas and retention times less than 1%, a peak resolution (RS) greater than 2.0 between two adjacent peaks for the three analytes, theoretical plate numbers (N) of at least 2000 for each peak, and USP tailing factors (T) less than 1.5. The authors concluded that all parameters were found in accordance with the acceptance criteria.

Sultana and coworkers (Sultana et al., 2010) developed an HPLC method for simultaneous analysis of diltiazem and nonsteroidal anti-inflammatory drugs (NSAIDs) in the bulk drug, tablet dosage forms, and human serum. System suitability was appraised by sixfold replicate analysis of the drugs dissolved in mobile phase. The authors presented in a table results from measurement of retention time, capacity factor (K), theoretical plate (N), resolution (R_S), and tailing factor (T) for each compound peak area. All values (except resolution for two drugs, which showed values between 1.5 and 2.0) were found to be in accordance with FDA specifications.

Rane and coworkers (2008) developed and validated a chiral HPLC method for separation of epinephrine enantiomers in the bulk drug. System suitability was determined by injecting a racemic mixture containing equal quantities of the D and L enantiomers. Because the enantiomers form a critical pair of peaks in the chromatogram, the authors established that resolution of the two enantiomers should not be less than 2.5 and the tailing factor should not exceed 1.5. The authors presented system suitability results in a table and concluded that all parameters were found in accordance with the acceptance criteria.

Rao and coworkers. (Rao et al., 2008) developed an HPLC method for determination of process impurities and degradation products of bicalutamide in bulk drug and pharmaceutical formulations. The system suitability was conducted using 2.5% (w/w) of all the impurities spiked to bicalutamide (200 μg mL^{-1}) and evaluated by making five replicate injections. According to the authors, the system was suitable for use if resolution for all

compounds was not less than 2.27. A minimum resolution of 2.27 was observed for all impurities, and the authors considered this minimum resolution as a system suitability parameter for the proposed method.

Solution Stability

To produce reliable and reproducible results, the samples, standards, and reagents must be stable for the period in which they will be stored. Frequently in automatic systems, the chromatographic injections are carried out overnight, which requires the solutions to be stable throughout this period. The stability of samples and standards should be tested over at least a 48 h storage period, after which the components should be quantified. In the case of instability over this period, storage conditions or additives that can improve stability should then be identified (Chandran and Singh, 2007).

As a statistical confirmation of stability, it is recommended to compare the area under the peak at the time when samples are first prepared to the area under the peak at time t of storage. This can be statistically confirmed by the regression relation $A_t = A_0 + bt + e$, where A_t is the area under the peak at time t, A_0 is the area at the initial moment, b is the slope of the regression line, and e is the random part of the model. For this statistic to follow Student's t distribution, it is necessary that e follows a normal distribution, with mean zero, equal variance for each value of time t, and independence from one time ti to another tj. A parameter $tb = |b|/SD_b$ (SD_b is the standard deviation of the slope) is then calculated and compared with the critical (tabulated) Student criterion t for the confidence probability of 99% and $n - 2$ degrees of freedom, where n is the number of experimental points. If tb is smaller than the tabulated t value, the coefficient b is not statistically significant and, therefore, the stability of the analyzed compound is confirmed (Épshtein, 2004).

However, to the best of our knowledge, in most studies involving analytical validation of chromatographic methods in pharmaceutical analysis, a statistical approach to confirmation of stability is not shown. Chitturi and coworkers (Chitturi et al., 2008) have published a validation of a gradient HPLC method for ten related substances determined in the lopinavir drug formulation. The authors determined lopinavir stability by spiking samples with related substances dissolved in a water and acetonitrile mixture (30:70 v/v). The solutions were analyzed immediately after preparation, and then after different time intervals up to 15 h, while maintaining samples at both 25°C and 6°C. The authors concluded from these studies that the sample solution, while unstable at room temperature, was stable for at least 15 h at 6°C.

Kasawar and Farooqui (2010) developed and validated an HPLC method for the quantification of related impurities of albuterol sulfate and ipratropium bromide in liquid pharmaceutical dosage form. The stability of drugs in analytical solution was checked by preparing sample solutions as per method and injecting at regular time intervals in the proposed method at room temperature. On verifying the formation of additional peaks, it was found that no additional peaks were formed until 26 h, indicating that the sample solution is stable over this period at room temperature.

Rao and coworkers (Rao et al., 2010) published a stability-indicating ultra-performance liquid chromatophaphic method for desloratadine and its impurities in pharmaceutical dosage forms. The stability of desloratadine in solution was determined by leaving test solutions of the sample and reference standard in tightly capped volumetric flasks at room temperature for 48 h during which they were assayed at 12 h intervals. Stability of mobile phase was determined in the same way; however, the mobile phase was prepared at the beginning of the study period and not changed during the experiment. The percentage assay of the results was calculated for both the mobile phase and solution-stability experiments. The stability of desloratadine and its impurities in solution for the related substance method was determined by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h and measuring the amounts of impurities every 12 h. The stability of mobile phase was also determined by analysis of a freshly prepared solution of desloratadine and its impurities at 12 h intervals for 48 h. The mobile phase was not changed during the study period. The authors concluded that standard solutions, sample solutions, and related substances were stable up to 48 h because assay (%) of desloratadine during solution stability and mobile phase stability experiments was within ±1% and the variability in the estimation of desloratadine impurities was within ±10% during solution stability and mobile phase experiments.

Kalíková and coworkers (Kalíková et al., 2008) published an HPLC method for the separation and quantification of cloprostenol enantiomers. Stability of the sample solutions was checked under various storage conditions for three weeks. Three equal solutions of (±)-cloprostenol were prepared by dilution of the stock solution with the mobile phase (to the final concentration of 0.1 mg/mL). The first sample solution was stored at low temperature (in the refrigerator), the second one at room temperature and in darkness, and the last solution was held also at room temperature but in daily light. The authors concluded from these studies that all the samples were proved to be stabile over the three-week period.

Khedr and Sheha (2008) developed a sensitive stability-indicating HPLC assay to study the stability of betahistine at different stress conditions. Betahistine and its decomposition products were derivatized by reaction with dansyl chloride and analyzed by HPLC equipped with a fluorescence detector. The authors tested sample solution stability by a repetitive daily injection of betahistine solution (1 ng μL^{-1}) and photodecomposed betahistine at 1.85 ng μL^{-1} for four days, and they concluded that these samples were stable at room temperature for 24 h. Moreover, the authors show that the samples left in the autosampler for more than two days have different results.

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However, the authors freshly prepared the reagent (dansyl chloride) every day, and they state that to apply this method successfully, a freshly prepared solution should be used and the analysis should be done within 12 h.

Selectivity

Some authors consider selectivity and specificity as different terms, as the term selective refers to a method that produces different responses for different chemical entities or analytes, while specificity is the ability to assess, unequivocally, the analyte in the presence of components that may eventually be found with the analyte (ICH, 2005). However, both selectivity and specificity are considered one in the same for other authors (Taverniers et al., 2004).

Selectivity can be assessed in several ways. The first is a comparison between a matrix without analyte and a matrix with this analyte added. In this case, the interfering compounds present should not affect the assay result (ICH, 2005). In HPLC methods, this parameter is frequently verified by an absence of interferences at the retention times of interest.

If a matrix without the analyte is not available, the selectivity determination can be carried out through a standard curve slope comparison of two analytical curves. One curve includes the sample matrix, and the other represents a sample without matrix. The selectivity is then assessed by comparing these two linear regression curve slopes. If they are similar, the method is considered selective and the matrix did not cause interference in the method (Bruce et al., 1998).

An additional way to assess selectivity is by the signal purity analysis measurement using mass or a UV/vis-diode array. In this approach, the drug substance or drug product is spiked with appropriate levels of impurities, and the signal purity is used to confirm that no other compounds co-elute with the analyte. When impurities are not available, the analyte can be exposed to severe stress conditions to force the creation of degradation products in a forced degradation study. The stress factors for bulk pharmaceutics forced degradation studies must include acid (0.1 Mol L^{-1} HCl) and base hydrolysis (0.1 Mol L^{-1} NaOH), thermal degradation (50°C), photolysis (600 foot candles), and oxidation (3% H_2O_2). Forced degradation studies for formulated products are often conducted under conditions such as heat (40°C), light, and humidity (60–75% RH) (Chandran and Singh, 2007).

An example of selectivity determination can be found in the work published by Ali and Nazzal (2009). The authors developed and validated a reversed-phase HPLC method for the simultaneous analysis of simvastatin (SIM) and tocotrienols (TRF) in nanoparticles. To confirm the selectivity of this method in regards to the excipients, the authors carried out a comparison between excipient chromatograms of nanoparticles containing TRF and SIM with TRF and SIM standard solutions. The method specificity, in regards to degradation products, was demonstrated by a comparison between reference chromatograms and the major SIM degradation product (i.e., de-esterified SIM). To convert SIM to its corresponding degradation product, the authors performed a forced degradation under basic hydrolytic conditions by incubating SIM in a 70% ethanol solution containing 0.1 Mol L^{-1} NaOH at 50°C for 1 h. The authors demonstrated the method selectivity by confirmation of the lack of any interference at the retention times of interest.

Bianchini and coworkers (Bianchini et al., 2009) developed and validated an HPLC method for the determination of process-related impurities in bulk drug of the central anticholinergic compound pridinol mesylate. The method selectivity was determined employing a diode-array detector under the optimized chromatographic conditions. According to the authors, all the analytes were well separated, with resolution higher than two between adjacent peaks. In addition, the peak purity function was employed and values of 0.9997, 0.9998, and 0.9996 for pridinol mesylate, 1-(3,3-diphenylprop-2-en-1-yl) piperidine, and 3-piperidinopropiophenone hydrochloride, respectively, excluded the presence of co-eluting interferences embedded in the peaks. Moreover, forced degradation samples (neutral, acid, basic, oxidative, and photolytic) produced no additional peaks. Based on these results, the authors confirmed the selectivity of the proposed method.

Tamaro and coworkers (Tamaro et al., 2010) developed and validated a stability-indicating HPLC method for the determination of alizapride and its main degradation products alizapride carboxylic acid (AL-CA) and alizapride N-oxide (AL-NO_2) in drug substance and product. To assess the method selectivity, the authors prepared tablets and ampoule formulations with the excipients used for commercial preparations. Moreover, to evaluate the influence of degradation products, a standard solution was prepared with the addition of the degradation products. After HPLC analyses, resolution factors were calculated. The authors demonstrated the method selectivity by confirmation of no peak interfering with analytes. Moreover, the adjacent chromatographic peaks were separated with resolution factors higher than five. The authors concluded from these data that the excipients and the degradation products did not interfere with the alizapride peak, indicating selectivity of the method.

Dencausse and coworkers (Dencausse et al., 2008) validated an HPLC method for quantitative determination of Tinosorb®S (bis-ethylhexyloxyphenol methoxyphenyl triazine) with three other sunscreen agents (benzophenone-3, butyl methoxydibenzoylmethane, and ethylhexyl methoxycinnamate) in a high-protection cosmetic product. The selectivity of the method was determined by comparing the results obtained from the analysis of a blank matrix solution with those of standard solution without matrix. According to the authors, the chromatogram of the blank matrix indicates that there is no analytical signal at the retention times of sunscreens and internal standard. Additionally, the selectivity of the method is shown by a satisfactory resolution greater than 1.25 for each peak.

Kafkala and coworkers (Kafkala et al., 2008) developed and validated an HPLC method for the determination of donepezil hydrochloride assay and impurities content in an oral

pharmaceutical formulation. The method selectivity was assessed by preparing donepezil hydrochloride solution, individual impurities solution, standard solution, sample solution, and placebo solution, which were analyzed by the proposed method. The spectra and purity plots were extracted through a diode array detector for each ingredient in the standard solution. Furthermore, the authors conducted forced degradation studies in order to prove selectivity of the method. The sample solution was subjected to acid and basic hydrolysis (using 1 Mol L^{-1} HCl and 1 Mol L^{-1} NaOH, respectively, for 2 h), to oxidative hydrolysis (using 30% H_2O_2 for 24 h), and to UV radiation (254 nm for 24 h). The authors show that donepezil hydrochloride and its impurities are well separated from each other in a sample solution injection and that the placebo solution shows no interfering peak with the rest of the analytes. Additionally, the authors show the purity angle values for all peaks derived from the purity plots, and they concluded that the obtained values were acceptable (purity angle < purity threshold in all cases). Furthermore, forced degradation studies confirmed the selectivity of the method. Donepezil showed degradation products after alkaline hydrolysis and oxidation, and the purity angle of the main peak maintained an acceptable value during all stress conditions.

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Linearity and Range

Linearity is the ability of an analytical procedure to obtain results that are directly proportional to the analyte concentration in the sample, within a given range (ICH, 2005). The mathematical correlation between obtained results and analyte concentration is represented by the calibration equation:

$$y = bx + a + \varepsilon \quad [6]$$

where y is the measured response, x is the analyte concentration, b is the calibration curve slope, a is the intercept, and ε is the random error term, the error in predicting the value of Y, given the value of X.

Frequently, linearity is judged through the correlation coefficient assessment, r (Equation (7)), and the linear regression y-intercept of the response versus concentration plot.

$$r = \sum\nolimits_i (xi - \overline{x})(yi - \overline{y}) \Big/ \sqrt{\sum\nolimits_i (xi - \overline{x})^2 \sum\nolimits_i (yi - \overline{y})^2} \quad [7]$$

where y is the measured response and x is the analyte concentration.

A high correlation coefficient means the data fit well with the regression line, and, for chromatographic methods, a correlation coefficient greater than 0.999 is generally considered acceptable (U.S. Food and Drug Administration, 2000). However, even such a high level of correlation may be accompanied by significant deviations from linearity in the regions of high and low drug concentrations (Épshtein, 2004). Therefore, before applying any inferential method, it is not recommended to pay attention only to the correlation coefficient itself. It is necessary to establish the following:

(i) The residuals must follow the same distribution (normal and homoscedastic), independent of the concentration (Analytical Methods Committee, 1994). Residuals are defined as the difference between calculated values $yi = a + bC$ from the measured Yi values as the function of the concentration Ci. A visual approach is useful to check if the residual values are randomly distributed about the regression line. Residuals examples plotted against concentrations in random and systematic distributions are shown in Figure 1.

(ii) The regression model should be tested, in both regression and in that there is not significant lack of fit (Analytical Methods Committee, 1994). To check the regression, the hypothesis is that the variation of y is explained by a regression model. In this approach, an F value is calculated by dividing the explained variance by the unexplained variance. When F is significant ($F_{calculated}$ is higher than the critical value F for 1 and n-2 degrees of freedom at risk level of 5% (n is the number of experimental points)), the regression equation helps us to understand the relationship between X and Y (Analytical Methods Committee, 1994). If the first hypothesis is acceptable, then a second hypothesis would be tested. If not, the regression model is not valid. The second hypothesis is to check the lack of fit of the model. In this approach, the F value is calculated on the basis of the ratio between the variance due to the linearity and that due to nonlinearity, and the variance due to the residue. The hypothesis that the regression model is linear can be accepted if $F_{calculated}$ is lower or equal to the critical value F for u-2 and n-u degrees of freedom at risk level of 5% (u is the number of concentration levels and n is the number of experimental points).

Moreover, a Student t test to decide if the intercept value ($P < 0.05$) is different from zero can be carried out, which is accomplished by obtaining a calculated t value ($t_{cal} = |a|/SD_a$, where a is the intercept and SD_a is the intercept standard deviation). If t_{cal} is lower than or equal to the critical value t distribution for n-2 degrees of freedom at the risk level of 0.05, the hypothesis of an intercept not significantly different from zero is accepted (Épshtein, 2004).

An additional way to assess linearity is by dividing the analytical signal by the respective concentrations, yielding relative responses. A graph is then plotted with the relative responses on the ordinate axis (y) and the corresponding concentrations on the abscissa axis (x), on a log scale. Parallel horizontal lines corresponding to 95% and 105% of the central horizontal line are drawn on the graph to establish a linear range limit. The central horizontal line corresponds to the average relative response. The method is linear in the range where the plotted relative responses are within the horizontal lines (Taverniers et al., 2004). Figure 2 illustrates a graphical evaluation using this approach.

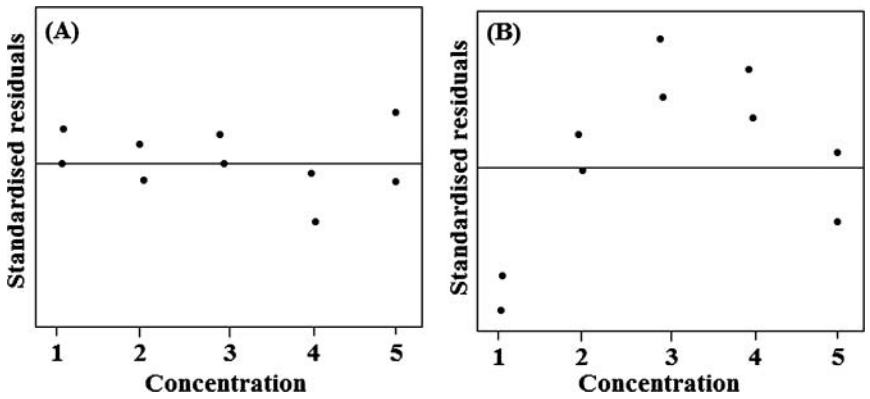

FIG. 1. Residuals examples plotted against concentrations in random and systematic distributions: (A) no convincing pattern in the residuals, and (B) a clear trend.

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In practice, calibration curves are constructed by using analytical solutions, with or without the sample matrix, and by diluting these solutions to different concentration levels covering the entire working range. The USP (*United States Pharmacopoeia*, 2009) and ICH (2005) recommend a minimum of five concentrations covering from 80 up to 120% of the test concentrations. On the other hand, IUPAC (Thompson, 2000) recommends six or more calibration standards over a concentration range of 0 to 150%, or 50 to 150% of the test concentration. The AOAC (2000) determined that four concentration levels should be selected (target concentrations of 50, 100, 150, and 200% of the test concentration) across three different days.

Work recently published by Mostafavi and coworkers (Mostafavi et al., 2009) presents an HPLC development and validation method for buprenorphine hydrochloride (BH), naloxone hydrochloride dehydrate (NH), and noroxymorphone (NM) determination in a tablet formulation. The method linearity was evaluated through standard calibration curves constructed with seven calibrators over a 0.22–220 μg mL^{-1} concentration range for BH and a 0.1–100 μg mL^{-1} range for both NH and NM. Three calibration curves were generated for each drug on three consecutive days ($n = 3$), and the values of evaluation parameters like slope, intercept, correlation coefficient, and squares residual sum are reported as the mean $\pm$ S.D. of the calibration curves.

A second example of linearity assessment that deserves to be cited is the work of Hoti and coworkers (Hoti et al., 2008). The authors published a reverse phase HPLC-MS method to assess the dissolution behavior of sugarcoated rociverine tablets. This method linearity was evaluated by preparing rociverine reference standard solutions in 0.01 Mol L^{-1} HCl at concentrations of 1.45, 2.90, 4.45, 7.25, 10.40, and 12.50 mg L^{-1} in triplicate. The linearity was evaluated by linear regression analysis, and the method was demonstrated to be linear with a 0.998 correlation coefficient. Additionally, the regression was checked by means of the analysis of variance (ANOVA), which showed significant linear regression for one and four degrees of freedom at risk level of 5% (Fcalculated $>$ Fcritical).

Manassra and coworkers (Manassra et al., 2010) have published an analytical method by HPLC for simultaneous analysis of pseudophedrine hydrochloride, codeine phosphate, and triprolidine hydrochloride in liquid dosage forms. Linearity of the method was evaluated by analyzing ten different concentrations in the range of 0.06–1.00 mg mL^{-1} for pseudophedrine hydrochloride, 0.02–1.0 mg mL^{-1} for codeine phosphate, and 0.0025–1.0 mg mL^{-1} of triprolidine hydrochloride. According to the authors, the results obtained showed the method linearity, with a correlation coefficient of 0.9996 for pseudophedrine hydrochloride, 0.9997 for codeine phosphate, and 0.9993 for triprolidine hydrochloride.

Salgado and coworkers (Salgado et al., 2009) developed and validated an HPLC method for the analysis of fleroxacin in bulk and pharmaceutical dosage forms. The linearity of the proposed method was checked by construction of standard curves, which were prepared using six standard concentrations in the range of 0.2–20 μg mL^{-1} for fleroxacin. The representative linear equation obtained by the authors was $y = 422.82x - 2.129$, with $r^2 = 1$. The RSD% values of the slope and Y intercept of the calibration curve were 1.3 and 2.0, respectively. From these

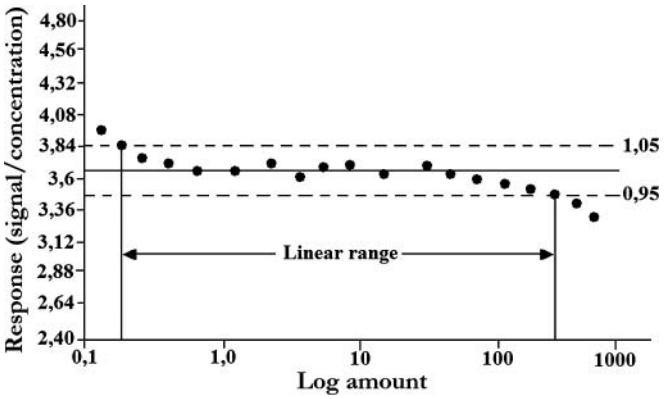

FIG. 2. Graph of a linear evaluation using an analytical signal/concentration ratio vs. concentration on a log scale.

results, the authors concluded that the results showed an excellent correlation existed between the peak area and concentration of the fleroxacin.

Precision

According to ICH (2005), analytical procedure precision is the closeness between multiple measurement series obtained from multiple sampling of the same homogeneous sample. This parameter is frequently evaluated in terms of the relative standard deviation estimative (see System Suitability section above) and may be considered at the levels of repeatability, intermediate precision, and reproducibility.

Repeatability (also termed intra-assay precision) expresses the precision under the same operating conditions using the same equipment over a short period of time (ICH, 2005). In practice, the same analyst would prepare nine samples (three low concentration replicates, three medium concentration replicates, and three high-concentration replicates) of a model mixture solution, a parent compound, or a drug, covering the rated range of concentrations. As there is strong evidence that repeatability in liquid chromatography is concentration dependent (Salgado et al., 2009), this approach is employed when the analytical method is intended to be used over a large concentration range.

Another way of determining repeatability is by analyzing repeated replicates of no less than six solution samples in concentrations close to the nominal value (ICH, 2005). IUPAC, however, states that at least seven solutions (preferably $n = 10$) should be analyzed to obtain good precision estimates (Thompson et al., 2000).

Intermediate precision (also termed inter-assay precision) characterizes the precision between results obtained in the same laboratory by different analysts using various instruments over at least two days. In this approach, no less than six independent solutions should be prepared at concentrations close to the nominal value (Épshtein, 2004). An interesting approach in intermediate precision assessment is to show that the average results (obtained with different analysts, over two or more days, or using various instruments) are statistically equivalent. According to Épshtein (2004), however, it is more convenient, in practical terms, to show that $RSD \leq 1.0\%$ for parent compound determination, $RSD \leq 2.0\%$ for drugs, and $RSD \leq 10.0\%$ for impurities.

Reproducibility validation is important if the method is going to be used in different laboratories. The IUPAC stated that it was not advisable to draw conclusions with fewer than five laboratories and generally calls for eight laboratories in its guidelines (Horwitz, 1994). Some studies in the literature evaluated reproducibility using eight or more laboratories by demonstrating the statistical equivalence between standard deviations of the obtained results (Horwitz, 1994; Hsu and Huang, 1992; Gill et al., 1986, 1989; Buchheit et al., 2002).

Bonifacio and coworkers (Bonifacio et al., 2009) presented an HPLC method validation for the determination of Cyclosporin A (CyA), four degradation products (ID-005-95, CyH, IsoCyH, and IsoCyA) and two related compounds (CyB and CyG) in pharmaceutical capsules. The precision of this method was assessed in terms of repeatability and intermediate precision. Repeatability was calculated from six replicate injections of freshly prepared solutions on the same equipment on the same day at 100% of the test concentrations. Impurities were tested at 2% of the CyA nominal concentration. This experiment was repeated at the same concentration on two additional and consecutive days to determine intermediate precision. Precision was expressed by percent relative standard deviation (RSD) of the analyte peak.

A second relevant example of precision determination is from Vignaduzzo and coworkers (Vignaduzzo et al., 2008). The authors developed and validated an RP-HPLC method for the simultaneous determination of meloxicam (MEL) and pridinol mesylate (PRI) in drug formulations. The intra-assay precision was evaluated by the injection of six independent samples at 100% level of the expected concentrations of the analytes in the tablet samples. The relative standard deviations observed were 0.4% and 1.0% for MEL and PRI, respectively. The intermediate precision was evaluated by means of a two-way ANOVA of recovery data of six standard independent mixtures injected by three independent analysts in triplicate across three different days. It was observed that all calculated F-ratios were smaller than the F-tabulated value, which led to the conclusion that there were no significant differences between days or analysts in the proposed method (Dencausse et al., 2008).

Kumar and coworkers (Kumar et al., 2009c) developed and validated a stability indicating HPLC method for quantification of exemestane and its impurities (process related and degradants). The assay method precision was evaluated by carrying out six independent assays of test samples of exemestane against qualified reference standard and calculated the percentage of relative standard deviation (% R.S.D.). The precision of the related substance was checked by injecting six individual preparations of exemestane spiked with 0.15% of impurities with respect to exemestane concentration; % R.S.D. of area for each impurity was then calculated. The intermediate precision of the method was evaluated using different analysts, on different days with different make instruments in the same laboratory. According to the authors, the % R.S.D. of assay of exemestane during the assay method precision study was within 0.6%. The % R.S.D. of area of impurities in the related substance method precision study was within 5%. The % R.S.D. of assay results obtained in the intermediate precision study was within 1.0%, confirming good precision of the method.

Gerpe and coworkers (Gerpe et al., 2008) developed an HPLC method for the determination of six antichagasic phenylethenylbenzofuroxans and their major synthetic secondary products. The method precision was established by injecting six standard samples of each active compound at three concentration levels for the intraday precision and on three days for the inter-day precision. Precision was expressed by the percentage of relative

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standard deviation of the analyte peaks. By applying a Fisher F-test for the ratio of variances, the authors concluded that no significant differences were observed between inter-day and intraday results for percentage of relative standard deviation.

Hadad and coworkers (Hadad et al., 2009) developed and validated a stability-indicating HPLC method for separation and quantitation of paracetamol, dantrolene, cetirizine, and pseudoephedrine in two pharmaceutical dosage forms. Repeatability and intermediate precision of the proposed method were performed at three concentration levels for each compound. The authors performed an 8 days $\times$ 2 replicates design, and a statistical comparison of the results was carried out using the P-value of the F-test. The authors concluded that there is no statistically significant difference between the mean results obtained from each day to another at the 95% confidence level, since the P-value of the F-test is always greater than 0.05, confirming, therefore, the method precision.

Accuracy

The accuracy, which can also sometimes be termed exactness, is the closeness of an obtained result to the true value. According to ICH (2005), accuracy should be established across the specified range of the analytical procedure. This parameter is expressed in terms of error or bias percentages.

There are several ways to determine the accuracy of an analytical method. When a certified reference material is available, the accuracy can be determined by comparison of the measured value with the declared true value. In this case, accuracy should be reported as the difference between the mean and the accepted true value together with the confidence intervals (ICH, 2005). Accuracy can also be obtained by comparison of the results of a proposed analytical procedure with those of a second, well-characterized reference method. The samples used in this approach may be certified reference materials, in-house standards, or analytical samples (Taverniers et al., 2004). When the certified reference material or method is not available, a recovery study could be performed. This procedure is carried out by spiking a known amount of analyte (by weight or volume) into a blank sample matrix. Recovery is then calculated according to:

$$R(\%) = \frac{(\text{found content})}{(C\text{added})} \times 100\% \qquad [8]$$

An alternative approach is the standard addition technique, which is used when it is not possible to prepare a blank sample matrix without the analyte being present. In this case, the recovery is calculated by:

$$R(\%) = \frac{(\text{C spiked measured} - \text{C unspiked measured})}{(C\text{added})} \times 100\% \qquad [9]$$

In all cases, an appropriate analyte concentration range should be investigated because the analyte recovery may be concentration dependent. At very low levels, the analyte may be largely chemisorbed on the analytical vessel matrix or surface, resulting in recoveries close to zero. At higher concentrations, however, where the adsorbed analyte is only a small fraction of the total analyte, the recovery may be effectively unchanged (Thompson et al., 2000).

According to ICH (2005), accuracy should be estimated at nine measurements for at least three different concentrations (three replicates of each: low, medium, and high concentration ranges). The FDA (U.S. Food and Drug Administration, 2000) recommends that accuracy should be estimated at three concentration levels for five replicates of each level.

The acceptable recovery percentage depends principally on the sample concentration range, the matrix, and the quality level required by the method. The following limits are acceptable: from 99.0 up to 101.0% for parent substance quantitative analysis with a high amount of active component (98% and above) and from 98.0 up to 102% for parent substance quantitative analysis with a lower amount of active component (98% and below). For ready-to-use drugs, these limits are: from 90.0 to 110% for quantitative determination of impurities with a rated maximum content of up to 1%, from 75 up to 125% for the quantitative determination of impurities with a rated content from 0.1 to 1%, and from 50.0 up to 150% for quantitative determination of impurities with a rated content below 0.1% (Épshtein, 2004).

An accuracy example in pharmaceutical product analytical validation is from Cazedey and coworkers (Cazedey et al., 2009). The authors published HPLC method development and validation for the analysis of ciprofloxacin hydrochloride in ophthalmic solutions. The accuracy was determined by adding known amounts of reference substance to the sample at three concentration levels. From this study, the authors found recovery values between 98.0 and 102%.

Garnero and Longhi (2010) developed an HPLC method for the determination of ascorbic acid using hydroxypropyl-β-cyclodextrin and triethanolamine as photostabilizing agents. The method accuracy was determined by analyzing three different concentrations of ascorbic acid through recovery studies. Three samples were prepared at each concentration level, and the recoveries of ascorbic acid were obtained by comparison of measured concentration with nominal concentration. The authors concluded that good recoveries of ascorbic acid were obtained at each concentration (within $100 \pm 2\%$), suggesting the accuracy of the proposed methods.

Jia and coworkers (Jia et al., 2010) published a method for determination of four kinds of anti-diabetic drug by HPLC with ultraviolet (UV) detection, evaporative light scattering detection (ELSD), and charged aerosol detection (CAD). The authors compare the results with reference to linearity, accuracy, precision, and limit of detection (DL). The accuracy was tested by injecting 10, 50, and 90 μg mL^{-1} of each standard into the detectors and comparing the peak areas with those from the calibration curves. For the recovery, 500 μg/mL of each standard was spiked to the methanol extract of a dietary supplement (product A, Canada) and the recovery was calculated

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according to the following equation: recovery (%) = experimental amount/spiked sample amount × 100. According to the authors, the accuracies of UV, CAD, and ELSD detection were all acceptable, with the values around 100%, and the% recovery of CAD was better than that of UV detection or ELSD.

Al-Rimawi (2009) developed and validated a stability-indicating HPLC method for the analysis of metformin hydrochloride and its related compound (1-cyanoguanidine) in tablet formulations. The accuracy of the proposed analytical method was studied by preparing the placebo of the drug formulation according to the formulation procedure. A known quantity of metformin hydrochloride with the same proportion as in the drug formulation was then added to get three concentrations (0.01, 0.02 (nominal concentration), and 0.03 mg mL^{-1}). The authors concluded that the accuracy of the current method is comparable to the accuracy of other methods listed in the references because they found mean recovery results of metformin hydrochloride within $100 \pm 2.0\%$, and an RSD lower than 1.0%.

Kumar and coworkers (Kumar et al., 2009b) developed and subsequently validated an HPLC method for the determination of almotriptan malate and its process-related impurities. The authors demonstrated the method accuracy by recovery test at four different concentration levels (25%, 50%, 100%, and 150% of the specification limit) in triplicate. The authors concluded that the method was found to be accurate, since the mean recoveries of all impurities were found to be in the range of 96–102%.

Detection and Quantitation Limit

Detection limit is the smallest measured concentration of an analyte from which it is possible to deduce the presence of the analyte in the test sample with acceptable certainty. Several approaches for determining the detection limit (DL) are possible. For analytical procedures that exhibit baseline noise, an alternative is based on the signal-to-noise ratio. This alternative approach is performed by comparing measured signals from blank samples with known low analyte concentrations, and then establishing the minimum concentration at which the analyte can be reliably detected. According to ICH (2005), a signal-to-noise ratio of between 3:1 or 2:1 is generally considered acceptable for estimating the detection limit.

However, the most common way for determining the detection limit is based on the slope (S) and on the standard deviation of the response at zero concentration level (σ). The slope may be estimated from the analyte calibration curve. However, it is recommended to determine the calibration curve slope using reference sample solutions with concentrations in the vicinity of the DL. The standard deviation value can be determined using the standard deviation of the area under the peak in the drug chromatograms (ten sequential injections) within 0.01–0.05% of the nominal drug concentration (Épshtein, 2004). An alternative is to construct several calibration curves in the DL region and determine the standard deviation of the intersections. Detection limit is then expressed by:

$$DL = \frac{3.3\sigma}{S} \quad [10]$$

where σ is the standard deviation of the responses and S is the calibration curve slope, which are calculated according to the above explanation.

Another way of determining DL is from the minimum concentration for which the RSD for the analyzed compound peak area for five sequential determinations does not exceed 20% (Épshtein, 2004).

The quantitation limit (QL) is the lowest amount of analyte in a sample that can be quantitatively determined with suitable accuracy (ICH, 2005). QL could be calculated based on the signal-to-noise ratio (S/N). According to this method, an initial reference solution of the analyzed compound is measured for which the signal-to-noise ratio is at least 30. Then this solution is sequentially diluted until this ratio decreases to $S/N \cong 10$. This corresponding concentration is then considered to be the QL (Épshtein, 2004). This procedure uses peak heights from HPLC (rather than areas), presenting a disadvantage in that it does not take into account the requirements of reproducibility of the HPLC procedure (Épshtein, 2004).

The QL could also be calculated based on the standard deviation of the responses and the slope. The quantitation limit is expressed by:

$$QL = \frac{10\sigma}{S} \quad [11]$$

where σ is the standard deviation of the responses and S is the calibration curve slope.

The slope and σ may be estimated in the same way as presented in the DL calculation, by using either the analyte calibration curve or a calibration curve at concentrations in the vicinity of the QL. Another method for determining QL is through the analysis of the minimum concentration for which six sequential injections of the analyzed compound give a RSD $\leq 2.0\%$ (Adamovics, 1997).

A practical example of the determination of detection and quantitation limits is from Shaikh and coworkers (Shaikh et al., 2008). The authors validated a reversed-phase HPLC method for the simultaneous estimation of ambroxol hydrochloride and azithromycin in tablets. DL and QL for analytes were estimated from calibration curves generated by triplicate injections of azithromycin and ambroxol hydrochloride solutions at concentrations in the vicinity of the DL and QL; QL and DL were then calculated by using Equations (10) and (11), respectively. The σ used by the authors was the regression line residual standard deviation.

Das Neves and coworkers (das Neves et al., 2010) developed and validated an HPLC method for the in vitro pharmaceutical characterization of dapivirine-loaded polymeric nanoparticles. DL and QL were determined based on the response and slope of a specific calibration curve obtained from six standard solutions

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(0.3, 0.4, 0.6, 0.8, 1.0, and 1.5 μg mL^{-1}) that were in proximity of these limit concentration values. Equations (10) and (11) were used by the authors. The σ used was the standard deviation of the responses, and S was the slope of the calibration curve.

Devi and Chandrasekhar (2009) validated a stability-indicating HPLC method for the quantitative determination of levofloxacin in the presence of degradation products and its process-related impurities in bulk samples and in pharmaceutical dosage forms. The DL and QL for levofloxacin impurities were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentration. Precision study was also carried at the level of quantification (LOQ) by injecting six individual preparations of the impurities and calculating the percentage RSD of the area.

Azeem and coworkers (Azeem et al., 2009) developed and validated a stability-indicating HPLC method for analysis of buspirone in the bulk drug and in solid dosage formulations. DL and QL were calculated from Equations (10) and (11), based on the standard deviation of the responses for blank injection in triplicate (σ) and the slope (S) of the calibration curve obtained in linearity study.

Ruggedness

The ruggedness of an analytical procedure is the capacity of its measurements to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage (ICH, 2005). Ruggedness is a term used by IUPAC (Thompson et al., 2000) and USP (*United States Pharmacopoeia*, 2009), but Eurachem (Eurachem Guide, 1998) and ICH (2005) have used the term "robustness," and, according to Eurachem, both parameters are synonymous.

Ruggedness is usually studied by varying typical parameters that are capable of influencing analysis results, such as the organic solvent content in the eluent (± 2%), amount of additives (salts, ion-pair reagents, etc.) in the eluent (± 10%), the buffer solution pH (± 0.5), HPLC column temperature (± 5°C), compound extraction time from a drug eluent (± 20%), extractant composition (± 5%), eluent concentration gradient (± 2%), mobile phase flow rate, column type, and/or manufacturer (Épshtein, 2004). It is desirable that the proposed analytical procedure is robust with respect to all these important parameters, which would make the given procedure suitable for routine laboratory use.

Narashiman and coworkers (Narashiman et al., 2009) described the development and validation of a stability-indicating reverse-phase HPLC method for oseltamivir active pharmaceutical ingredient analysis. Method ruggedness was investigated by varying the chromatographic conditions, such as flow rate (± 10%), organic content in mobile phase (± 2%), detection wavelength (± 5%), and buffer pH in mobile phase (± 0.2%). The developed method ruggedness was indicated by the overall% RSD < 2 between the data at each variable condition.

Srinivasu and coworkers (Srinivasu et al., 2010) developed and validated a stability-indicating HPLC method for the quantitative determination of acetazolamide and its related substances. To determine the ruggedness of the developed method, the authors altered the experimental conditions and evaluated the resolution between acetazolamide and its impurities. The flow rate of the mobile phase was 1.0 mL/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.2 units (0.8 and 1.2 mL/min). The effect of pH on the resolution of the impurities was studied by varying the pH by ±0.1 units (buffer pH of 2.9 and 3.1). The effect of the column temperature on the resolution was studied at 22°C and 32°C instead of 27°C. The authors concluded that in all of the deliberately varied chromatographic conditions carried out, the resolution between the closely eluting impurities was greater than 5.0, illustrating the ruggedness of the method.

An alternative ruggedness assessment is by the multivariate approach. Youden and Steiner (1975) proposed a test where the basic idea is to introduce several changes at once in such a manner that the individual effects can be ascertained. This test consisted of the evaluation of seven variables that could influence the final result if their nominal values were slightly changed. Each variable effect was investigated at two levels, as indicated by upper- (A, B, C, D, E, F, and G) and lowercase (a, b, c, d, e, f, and g) letters. Uppercase letters represented high-level variable values and lowercase letters represents low-level variable values. From these results, it was possible to estimate each variable effect by obtaining the mean difference of four analyses that have a nominal value (capital letter) and the mean of four analyses with the alternative value (small letter). If the difference was higher than the standard deviation, the variable would have a significant effect and the method would be sensitive to changes in that variable.

A practical example of the Youden and Steiner test is from the work of Mirza and Tan (2001). The authors developed and validated an anion-exchange HPLC method for the analysis of captopril in tablet dosage form using indirect photometric detection. The ruggedness of this method was evaluated by varying mobile phase pH, mobile phase flow rate, marker concentration,% organic modifier, column temperature, detector wavelength, and column age. A total of eight experiments were conducted to identify variables that may have to be controlled to obtain accurate assay results, and their findings were designated by the letters s through z. From the results of these eight experiments, the effect of each variable could be determined, with the differences obtained between the two levels of each variable ranked in decreasing order. The obtained results indicated that the mobile phase pH was the most important variable that needed to be controlled to maintain accuracy.

An interesting multivariate approach for ruggedness assessment is presented by Ashenafi and coworkers (Ashenafi et al., 2010). The authors developed an HPLC method for the determination of oxytocin and its related substances in bulk drugs. In the ruggedness study, the influence of four chromatographic parameters on the separation was investigated. The parameters examined were the amount of acetonitrile in mobile phase, the pH of the dihydrogen phosphate solution, the amount of

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dihydrogen phosphate solution in the mobile phases, and the column temperature (°C). Their effects on the resolution of different peak pairs were evaluated by means of an experimental design and multivariate data analysis. The authors presented in a figure the calculated effects for each variable on the peak resolutions and the statistical errors. When the effects exceed the error, this effect becomes statistically significant. Furthermore, factors with positive effects indicate that an increase of their level provides a significant increase in the analytical response, and negative effects mean that a higher resolution between the peaks' value is obtained with a decrease in the levels of the factors. The authors observed that, under the conditions examined, the pH has a significant effect on the resolution of two peak pairs. The authors also discuss the influence of each chromatographic parameter on the separation, and they concluded that within the domain examined, the method can be considered robust, except for the pH, which has quite some influence and has to be set carefully.

Another experimental design often used in ruggedness testing is the Plackett-Burman design (1946). In this approach, N-1 factors (variables) are studied in N experimental runs (with N being a multiple of 4). The construction of these designs has been described in the literature (Plackett and Burman, 1946; Vander Heyden et al., 2001). The factors are investigated at two levels: (−) and (+). From the obtained responses, factor effects are calculated according to:

$$E_X = \frac{\Sigma Y(+) - \Sigma Y(-)}{N/2} \qquad [12]$$

where E_X is the effect of factor X, $\sum Y(+)$ and $\sum Y(-)$ the sums of the responses where factor X is at (+) or (−) level, respectively, and N is the number of design experiments. To identify significant effects, both graphical and statistical interpretation methods are described (Vander Heyden and Massart, 1996; Dejaegher et al., 2006, 2007).

A practical example of the Plackett-Burman design for ruggedness assessment is from the work of Halabi and coworkers (Halabi et al., 2004). The authors developed and validated a chiral HPLC method for determination of salbutamol enantiomers. The ruggedness of this method was evaluated by studying the effect of variation in the mobile phase acidity, mobile phase flow rate, and column temperature on the resolution, on the R.S.D. (%) of the *R*-SS peak area, and on the *R*/*S* ratio (ratio between peak areas of (R)-salbutamol sulfate and (*S*)-salbutamol sulfate). The variables were evaluated in four experiments, according to the combinations presented by the authors in a matrix, which was described by Plackett and Burman (1946). The effect of each factor was then calculated according to Equation (12). Values higher than the criterion $s(2)^{1/2}$ were considered important, where s is the standard deviation obtained in the precision study for each parameter. From the obtained results, the authors concluded that resolution, R.S.D. (%) of the *R*-SS peak area, and *R*/*S* ratio were almost unaffected by the considered factors, under the conditions studied.

CONCLUSION

Analytical method validation is a very important tool for ensuring reliable and accurate method performance. Although various regulatory bodies have already considered this subject, and a significant amount of advice exists in the literature, uniformity is still lacking in the methodology employed for validation and acceptance criteria. In practice, a laboratory must be able to decide the best method for carrying out the validation tests. For this reason, this review is useful as a guide to analytical laboratories because it offers several literature examples and presents relevant information for various parameters in the field of quantitative HPLC analytical methods validation in pharmaceutical analysis.

REFERENCES

Adamovics, J. *Chromatographic Analysis of Pharmaceuticals*; Marcel Dekker: New York, 1997.

Al-Rimawi, F. Development and Validation of an Analytical Method for Metformin Hydrochloride and Its Related Compound (1-Cyanoguanidine) in Tablet Formulations by HPLC-UV. *Talanta* **2009**, *79*, 1368–1371.

Ali, H.; Nazzal, S. Development and Validation of a Reversed-Phase HPLC Method for the Simultaneous Analysis of Simvastatin and Tocotrienols in Combined Dosage Forms. *J. Pharm. Biomed. Anal.* **2009**, *49*, 950–956.

Analytical Methods Committee. Is My Calibration Linear? *Analyst* **1994**, *119*, 2363–2366.

AOAC International. Method Validation Programs (OMA/PVM Department), including Appendix D: Guidelines for collaborative study procedures to validate characteristics of a method of analysis, 2000. http://www.aoac.org/Official_Methods/Collaborative_Study_Validation_Guidelines.pdf (accessed December 1, 2011)

Ashenafi, D.; Hemelrijck, E. V.; Chopra, S.; Hoogmartens, J. Liquid Chromatographic Analysis of Oxytocin and Its Related Substances. *J. Pharm. Biomed. Anal.* **2010**, *51*, 24–29.

Azeem, A.; Rizwan, M.; Ahmad, F. J.; Iqbal, Z.; Khar, R. K.; Aqil, M.; Talegaonkar, S. Development and Validation of a Stability-Indicating LC–UV Method for Rapid Analysis of Buspirone in Pharmaceutical Dosage Forms. *Acta Chromatogr.* **2009**, *21*, 283–297.

Bianchini, R. M.; Castellano, P. M.; Kaufman, T. S. Development and Validation of an HPLC Method for the Determination of Process-Related Impurities in Pridinol Mesylate, Employing Experimental Designs. *Anal. Chim. Acta* **2009**, *654*, 141–147.

Bonfilio, R.; Tarley, C. R. T.; Pereira, G. R.; Salgado, H. R. N.; Araújo, M. B. Multivariate Optimization and Validation of an Analytical Methodology by RP-HPLC for the Determination of Losartan Potassium in Capsules. *Talanta* **2009**, *80*, 236–241.

Bonifacio, F. N.; Giocanti, M.; Reynier, J. P.; Lacarelle, B.; Nicolay, A. Development and Validation of HPLC Method for the Determination

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of Cyclosporin A and Its Impurities in Neoral® Capsules and Its Generic Versions. *J. Pharm. Biomed. Anal.* **2009**, *49*, 540–546.

Bruce, P.; Minkkinen, P.; Riekkola, M. L. Practical Method Validation: Validation Sufficient for an Analysis Method. *Microchim. Acta* **1998**, *128*, 93–106.

Buchheit, K. R.; Daas, A.; Jönsson, K. H. Collaborative Study for Establishment of an HPLC-Method for Batch Consistency Control of Recombinant Interferon-Alfa-2. *Pharmeuropa Spec*. Issue Biol. **2002**, *1*, 7–27.

Cazedey, E. C. L.; Perez, D. P.; Perez, J. P.; Salgado, H. R. N. LC Assay for Ciprofloxacin Hydrochloride Ophthalmic Solution. *Chromatographia* **2009**, *69*, S241–S244.

Chandran, S.; Singh, R. S. P. Comparison of Various International Guidelines for Analytical Method Validation. *Pharmazie* **2007**, *62*, 4–14.

Chitturi, S. R.; Bharathi, C.; Reddy, A. V. R.; Reddy, K. C.; Sharma, H. K.; Handa, V. K.; Dandala, R.; Bindu, V. H. Impurity Profile Study of Lopinavir and Validation of HPLC Method for the Determination of Related Substances in Lopinavir Drug Substance. *J. Pharm. Biomed. Anal.* **2008**, *48*, 1430–1440.

das Neves, J.; Sarmento, B.; Amiji, M. M.; Bahia, M. F. Development and Validation of a Rapid Reversed-Phase HPLC Method for the Determination of the Non-Nucleoside Reverse Transcriptase Inhibitor Dapivirine from Polymeric Nanoparticles. *J. Pharm. Biomed. Anal*. **2010**, *52*, 167–172.

Dejaegher, B.; Capron, X.; Smeyers-Verbeke, J.; Vander Heyden, Y. Randomization Tests to Identify Significant Effects in Experimental Designs for Robustness Testing. *Anal. Chim. Acta* **2006**, *564*, 184–200.

Dejaegher, B.; Dumarey, M.; Capron, X.; Bloomfield, M. S.; Vander Heyden, Y. Comparison of Plackett–Burman and Supersaturated Designs in Robustness Testing. *Anal. Chim. Acta* **2007**, *595*, 59–71.

Dencausse, L.; Galland, A.; Clamou, J. L.; Basso, J. Validation of HPLC Method for Quantitative Determination of Tinosorb_S and Three Other Sunscreens in a High Protection Cosmetic Product. *Int. J. Cosmet. Sci.* **2008**, *30*, 373–382.

Devi, M. L.; Chandrasekhar, K. B. A Validated Stability-Indicating RP-HPLC Method for Levofloxacin in the Presence of Degradation Products, Its Process Related Impurities and Identification of Oxidative Degradant. *J. Pharm. Biomed. Anal.* **2009**, *50*, 710–717.

Épshtein, N. A. Validation of HPLC Techniques for Pharmaceutical Analysis. *Pharm. Chem. J.* **2004**, *38*, 212–228.

Épshtein, N. A.; Emshanova, S. V. Requirements to HPLC Systems Suitable for Quality Control of Parent Substances and Dosage Forms. *Pharm. Chem. J*. **2008**, *42*, 34–40.

Eurachem Guide. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics, 1998. http://www.eurachem.org/guides/valid.pdf (accessed December 1, 2011).

Fu, Q.; Shou, M.; Chien, D.; Markovich, R.; Rustum, A. M. Development and Validation of a Stability-Indicating RP-HPLC Method for Assay of Betamethasone and Estimation of Its Related Compounds. *J. Pharm. Biomed. Anal.* **2010**, *51*, 617–625.

Garnero, C.; Longhi, M. Development of HPLC and UV Spectrophotometric Methods for the Determination of Ascorbic Acid Using Hydroxypropyl-β-Cyclodextrin and Triethanolamine as Photostabilizing Agents. *Anal. Chim. Acta* **2010**, *659*, 159–166.

Gerpe, A.; Merlino, A.; Boiani, M.; Porcal, W.; Fagiolino, P.; González, M.; Cerecetto, H. Development of a HPLC Method for the Determination of Antichagasic Phenylethenylbenzofuroxans and Its Major Synthetic Secondary Products in the Chemical Production Processes. *J. Pharm. Biomed. Anal.* **2008**, *47*, 88–94.

Gill, R.; Moffat, A. C.; Smith, R. M.; Hurdley, T. G. A Collaborative Study to Investigate the Retention Reproducibility of Barbiturates in HPLC with a View to Establishing Retention Databases for Drug Identification. *J. Chromatogr. Sci*. **1986**, *24*, 153–159.

Gill, R.; Osselton, M. D.; Smith, R. M. International Collaborative Study of the Retention Reproducibility of Basic Drugs in High-Performance Liquid Chromatography on a Silica Column with a Methanol-Ammonium Nitrate Eluent. *J. Pharm. Biomed. Anal*. **1989**, *7*, 447–457.

Hadad, G. M.; Emara, S.; Mahmoud, W. M. M. Development and Validation of a Stability-Indicating RP-HPLC Method for the Determination of Paracetamol with Dantrolene or/and Cetirizine and Pseudoephedrine in Two Pharmaceutical Dosage Forms. *Talanta* **2009**, *79*, 1360–1367.

Halabi, A.; Ferrayoli, C.; Palacio, M.; Dabbene, V.; Palacios, S. Validation of a Chiral HPLC Assay for (*R*)-Salbutamol Sulfate. *J. Pharm. Biomed. Anal*. **2004**, *34*, 45–51.

Horwitz, W. Nomenclature of Interlaboratory Analytical Studies. *Pure Appl. Chem.* **1994**, *66*, 1903–1911.

Hoti, E.; Censi, R.; Ricciutelli, M.; Malaj, L.; Barboni, L.; Martelli, S.; Valleri, M.; Martino, P. D. Validation of an HPLC–MS Method for Rociverine Tablet Dissolution Analysis. *J. Pharm. Biomed. Anal*. **2008**, *47*, 422–428.

Hsu, M. C.; Huang, W. F. Collaborative Study of the Determination of Cloxacillin by Column Liquid Chromatography. *J. Chromatogr. A* **1992**, *600*, 333–336.

ICH Q2(R1). *Validation of Analytical Procedures: Text and Methodology*; ICH: Geneva, Switzerland, 2005.

International Organization for Standardization. *ISO 8402:1994, Quality – Vocabulary*; ISO: Geneva, Switzerland, 2005.

Jain, R.; Wu, Z.; Tucker, I. G. A Stability-Indicating HPLC Assay with Diode Array Detection for the Determination of a Benzylpenicillin Prodrug in Aqueous Solutions. *J. Pharm. Biomed. Anal.* **2009**, *50*, 841–846.

Jia, S.; Lee, W. J.; Ee, J. W.; Park, J. H.; Kwon, S. W.; Lee, J. Comparison of Ultraviolet Detection, Evaporative Light Scattering Detection and Charged Aerosol Detection Methods for Liquid-Chromatographic Determination of Anti-Diabetic Drugs. *J. Pharm. Biomed. Anal.* **2010**, *51*, 973–978.

Kafkala, S.; Matthaiou, S.; Alexaki, P.; Abatzis, M.; Bartzeliotis, A.; Katsiabani, M. New Gradient High-Performance Liquid Chromatography Method for Determination of Donepezil Hydrochloride Assay and Impurities Content in Oral Pharmaceutical Formulation. *J. Chromatogr. A* **2008**, *1189*, 392–397.

Kalíková, K.; Tesařová, E.; Bosáková, Z. HPLC Method For Enantioselective Analysis Of Cloprostenol. *J. Pharm. Biomed. Anal.* **2008**, *46*, 892–897.

Karakuş, S.; Küçükgüzel, I.; Küçükgüzel, Ş. G. Development and Validation of a Rapid RP-HPLC Method for the Determination of Cetirizine or Fexofenadine with Pseudoephedrine in Binary Pharmaceutical Dosage Forms. *J. Pharm. Biomed. Anal.* **2008**, *46*, 295–302.

Kasawar, G. B.; Farooqui, M. Development and Validation of a Stability Indicating RP-HPLC Method for the Simultaneous Determination of Related Substances of Albuterol Sulfate and Ipratropium Bromide in Nasal Solution. *J. Pharm. Biomed. Anal*. **2010**, *52*, 19–29.

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Khedr, A.; Sheha, M. Stress Degradation Studies on Betahistine and Development of a Validated Stability-Indicating Assay Method. *J. Chromatogr. B* **2008**, *869*, 111–117.

Kumar, R. S.; Naidu, M. N.; Srinivasulu, K.; Sekhar, K. R.; Veerender, M.; Srinivasu, M. K. Development and Validation of a Stability Indicating LC Method for the Assay and Related Substances Determination of Exemestane, an Aromatase Inhibitor. *J. Pharm. Biomed. Anal.* **2009a**, *50*, 746–752.

Kumar, R. S.; Naidu, M. N.; Srinivasulu, K.; Sekhar, K. R.; Veerender, M.; Srinivasu, M. K Development and Validation of a Stability Indicating LC Method for the Assay and Related Substances Determination of Exemestane, an Aromatase Inhibitor. *J. Pharm. Biomed. Anal.* **2009b**, *50*, 746–752.

Kumar, R. S.; Naidu, M. N.; Srinivasulu, K.; Sekhar, K. R.; Veerender, M.; Srinivasu, M. K. Development and Validation of a Stability Indicating LC Method for the Assay and Related Substances Determination of Exemestane, an Aromatase Inhibitor. *J. Pharm. Biomed. Anal.* **2009c**, *50*, 746–752.

Mahadik, M.; Bhusari, V.; Kulkarni, M.; Dhaneshwar, S. LC–UV and LC–MS Evaluation of Stress Degradation Behaviour of Tenatoprazole. *J. Pharm. Biomed. Anal.* **2009**, *50*, 787–793.

Manassra, A.; Khamis, M.; el-Dakiky, M.; Abdel-Qader, Z.; Al-Rimawi, F. Simultaneous HPLC Analysis of Pseudophedrine Hydrochloride, Codeine Phosphate, and Triprolidine Hydrochloride in Liquid Dosage Forms. *J. Pharm. Biomed. Anal.* **2010**, *51*, 991–993.

Mirza, T.; Tan, H. S. I. Determination of Captopril in Pharmaceutical Tablets by Anion-Exchange HPLC Using Indirect Photometric Detection; A Study in Systematic Method Development. *J. Pharm. Biomed. Anal.* **2001**, *25*, 39–52.

Mostafavi, A.; Abedi, G.; Jamshidib, A.; Afzali, D.; Talebi, M. Development and Validation of a HPLC Method for the Determination of Buprenorphine Hydrochloride, Naloxone Hydrochloride and Noroxymorphone in a Tablet Formulation. *Talanta* **2009**, *77*, 1415–1419.

Narasimhan, B.; Abida, K.; Srinivas, K. Stability Indicating RP-HPLC Method Development and Validation for Oseltamivir API. *Chem. Pharm. Bull*. **2008**, *56*, 413–417.

Plackett, R. L.; Burman, J. P. The Design of Optimum Multifactorial Experiments. *Biometrika* **1946**, *33*, 305–325.

Rane, V. P.; Patil, K. R.; Sangshetti, J. N.; Yeole, R. D.; Shinde, D. B. Enantiomeric LC Separation of Epinephrine on Amylose Based Sorbent. *Chromatogr.* **2008**, *67*, 777–781.

Rane, V. P., Patil, K. R., Sangshetti, J. N.; Yeole, R. D.; Shinde, D. B. Stability-Indicating RP-HPLC Method for Analysis of Eplerenone in the Bulk Drug and in a Pharmaceutical Dosage Form. *Acta Chromatogr*. **2009**, *21*, 619–629.

Rao, R. N.; Raju, A. N.; Narsimha, R. Isolation and Characterization of Process Related Impurities and Degradation Products of Bicalutamide and Development of RP-HPLC Method for Impurity Profile Study. *J. Pharm. Biomed. Anal.* **2008**, *46*, 505–519.

Rao, D. D.; Satyanarayana, N. V.; Reddy, A. M.; Sait, S. S.; Chakole, D.; Mukkanti, K. A Validated Stability-Indicating UPLC Method for Desloratadine and Its Impurities in Pharmaceutical Dosage Forms. *J. Pharm. Biomed. Anal*. **2010**, *51*, 736–742.

Salgado, H. R. N.; Moreno, A.; Garg, S. LC-DAD Determination of Fleroxacin in Bulk and Pharmaceutical Dosage Forms. *Chromatographia* **2009**, *69*, S237–S240.

Schepersa, U.; Ermerb, J.; Preua, L.; Wätzig, H. Wide Concentration Range Investigation of Recovery, Precision and Error Structure in Liquid Chromatography. *J. Chromatogr. B* **2004**, *810*, 111–118.

Shabir, G. A. Validation of High-Performance Liquid Chromatography Methods for Pharmaceutical Analysis. Understanding the Differences and Similarities between Validation Requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *J. Chromatogr. A* **2003**, *987*, 57–66.

Shaikh, K. A.; Patil, S. D.; Devkhile, A. B. Development and Validation of a Reversed-Phase HPLC Method for Simultaneous Estimation of Ambroxol Hydrochloride and Azithromycin in Tablet Dosage Form. *J. Pharm. Biomed. Anal.* **2008**, *48*, 1481–1484.

Shou, M.; Galinada, W. A.; Wei, Y. C.; Tang, Q.; Markovich, R. J.; Rustum, A. M. Development and Validation of a Stability-Indicating HPLC Method for Simultaneous Determination of Salicylic Acid, Betamethasone Dipropionate and Their Related Compounds in Diprosalic Lotion®. *J. Pharm. Biomed. Anal.* **2009**, *50*, 356–361.

Srinivasu, P.; SubbaRao, D. V.; Vegesna, R. V. K.; Babu, K. S. A Validated Stability-Indicating LC Method for Acetazolamide in the Presence of Degradation Products and Its Process-Related Impurities. *J. Pharm. Biomed. Anal.* **2010**, *52*, 142–148.

Sultana, N.; Arayne, M. S.; Shafi, N.; Siddiqui, F. A. Simultaneous RP-LC Analysis of Diltiazem and Non-Steroidal Anti-Inflammatory Drugs in Pharmaceutical Formulations and Human Serum. *Chromatographia* **2010**, *71*, 71–77.

Tamaro, I.; Aprile, S.; Giovenzana, G. B.; Grosa, G. Development and Validation of a Stability-Indicating HPLC-UV Method for the Determination of Alizapride and Its Degradation Products. *J. Pharm. Biomed. Anal.* **2010**, *51*, 1024–1031.

Taverniers, I.; Loose, M. D.; Bockstaele, E. V. Trends in Quality in the Analytical Laboratory. II. Analytical Method Validation and Quality Assurance. *Trends Anal. Chem.* **2004**, *23*, 535–552.

Thompson, M.; Ellison, S. L. R.; Wood, R. Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis. *Pure Appl. Chem.* **2000**, *74*, 835–855.

U.S. Food and Drug Administration. *FDATechnical Review Guide*; Center for Drug Evaluation and Research (CDER), Guidance for Industry, Analytical Procedures and Methods Validation, Chemistry Manufacturing and Controls Documentation, Draft Guidance, 2000. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122858.pdf.

The United States Pharmacopoeia. United States Pharmacopoeial Convention: Rockville, Md., 2009.

Vander Heyden, Y.; Massart, D. L. In *Robustness of Analytical Chemical Methods and Pharmaceutical Technological Products*; Hendriks, M. W. B., de Boer, J. H., Smilde, A. K., Eds.; Elsevier: Amsterdam, 1996; pp. 79–147.

Vander Heyden, Y.; Nijhuis, A.; Smeyers-Verbeke, J.; Vandeginste, B. G. M.; Massart, D. L. Guidance for Robustness/Ruggedness Tests in Method Validation. *J. Pharm. Biomed. Anal.* **2001**, *24*, 723–753.

Vignaduzzo, S. E.; Castellano, P. M.; Kaufman, T. S. Method Development and Validation for the Simultaneous Determination of Meloxicam and Pridinol Mesylate Using RP-HPLC and Its Application in Drug Formulations. *J. Pharm. Biomed. Anal.* **2008**, *46*, 219–225.

WHO. *WHO Expert Committee Report on Specifications for Pharmaceutical Preparations: Thirty-Second Report, 117*; WHO: Geneva, Switzerland, 1992.

Youden, W. J.; Steiner, E. H. *Statistical Manual of AOAC—Association of Offcial Analytical Chemistry*; AOAC: Washington, D.C., 1975.