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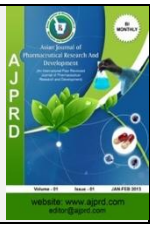

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Review Article

Simultaneous Estimation of Admixture drugs by RP-HPLC: A Review

Shipra Saini, Sulekha Mandal, Dilip Agarwal

Mahatma Gandhi College of Pharmaceutical Sciences, Jaipur, Rajasthan, India

A B S T R A C T

Recently, high demand of high-throughput analyses with high sensitivity and selectivity to molecules and drugs in different classes with different physical-chemical properties and a reduction in analysis time—is a principal milestone for novel methodologies that researchers are trying to achieve especially when analytical procedures are applied to clinical purposes. In addition, to avoid high doses of a single drug that could cause serious side effects, multi-drug therapies are often used to treat numerous diseases. For these reasons, the demand for methods that allow the rapid analysis of mixed compounds has increased in recent years. In order to respond to these needs, new methods and instruments have been developed. However, often the complexity of a matrix can require a long time for the preparation and processing of the samples. Different problems in terms of components, types of matrices, compounds and physical-chemical complexity are encountered when considering drugs association profiles for quantitative analyses. This review addresses not only recently optimized procedures such as chromatographic separation, but also methods that have allowed us to obtain accuracy (precision and trueness), sensitivity and selectivity in quantitative analyses for cases of drug associations.

Keywords: Analytical methods; chromatographic procedures; drug associations; hyphenated techniques; biologic matrices.

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***Address for Correspondence:**

Shipra Saini, Mahatma Gandhi College of Pharmaceutical Sciences, Jaipur, Rajasthan, India

INTRODUCTION:

The existing analytical approaches and instrumentation are usually tailored by the development of any new or improved method for the analysis of an analyte. Method development usually requires selecting the method requirements and deciding the type of instrumentation[1]. Decision regarding the choice of column, mobile phase detector and method of quantitation must be addressed in the development stage of an HPLC method.

It is important to determine chromatographic parameters for the analyte interest when once the instrumentation has been selected. The properties of the analytes that may be advantageous to select the nature of the column, establishing the approximate composition and pH of the mobile phase for separation, wave length to be employed or mass/charge ratio to be scanned at for detection of the compound, the concentration range to be followed and choice of a suitable internal standard for quantification purpose etc. are necessarily considered. Such information may already be available in the literature for the analyte or related compounds.

The above can be followed by optimization and preliminary evaluation of the method. Optimization criteria must be determined with cognizance of the goals common to any novel method. Initial analytical parameters of merit like sensitivity (measured as response per amount injected), limit of detection, limit of quantitation and linearity of calibration plots. It is important that method development is to be performed as a precautionary measure, in which the method development should be performed using only the analytical standards that have been well identified, characterized and whose purity is known.

The initial sets of conditions that have involved from the first stages of development are improved during the optimization stage. They are improved or optimized in terms of resolution, peak shape, plate counts, peak assymetry, capacity, elution time, detection limits, limits of quantitation and overall ability to quantify the specific analyte of interest. During optimization the results obtained must be evaluated against the goals of the analysis set forth by the analytical figures of merit. Additional improvement

and optimization which are needed to meet some of the initial method requirements may be revealed during this evaluation.

Maximum sensitivity, good peak symmetry, minimum detection and quantitation levels, a wide linearity range and a high degree of accuracy and precision should come from the optimization of the method. Base line resolution of the analyte of interest from other sample components, unique peak identification, online demonstration of purity and interfacing of computerized data for routine sample analysis comes under additional potential optimization goals. However, an absolute quantitation should use simplified methods that require minimal sample handling and analysis time.

Optimization of the method can follow either manual or computer driven approaches. The manual approach involves varying of one experimental condition at a time, while holding all others constant and recording changes in response. Flow rate, mobile or stationary phase composition, temperature, detection wavelength and pH may include in the variables. This univariate approach to system optimization is time consuming, slow and expensive. Nevertheless, it may provide a much better understanding of the principle involved and of the interactions of the variables. Efficiency is optimized where experimental input is minimised in computer driven automated method development. Computer driven automated approaches can be applied to many applications. Moreover, these computers driven automated approaches are capable of significantly reducing the cost of analysis, time and energy.

HPLC INSTRUMENTATION[2]

The HPLC instrumentation involves pump, injector, column, detector, integrator and display system. In the column the separation occurs.

The main parts of HPLC are:

a) Solvent or mobile phase reservoir
b) High pressure pump
c) Injector
d) Column
e) Detector

Solvent or Mobile phase reservoir:

The solvents or buffers or mixture of solvents and buffers in the form of homogenous mixture are stored in solvent reservoir. They are allowed to enter the mixing chamber through the mechanical pump viaflowing tube reservoirs that are mainly made up of glass bottled with properly covered.

High pressure pump:

The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.

Sample Injector:

The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Columns:

Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters of < 2 mm are regularly alluded to as microbore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.

Detector:

The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, massspectrometric and electrochemical identifiers. Data Collection Devices or Integrator: Signals from the detector might be gathered on graph recorders or• electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

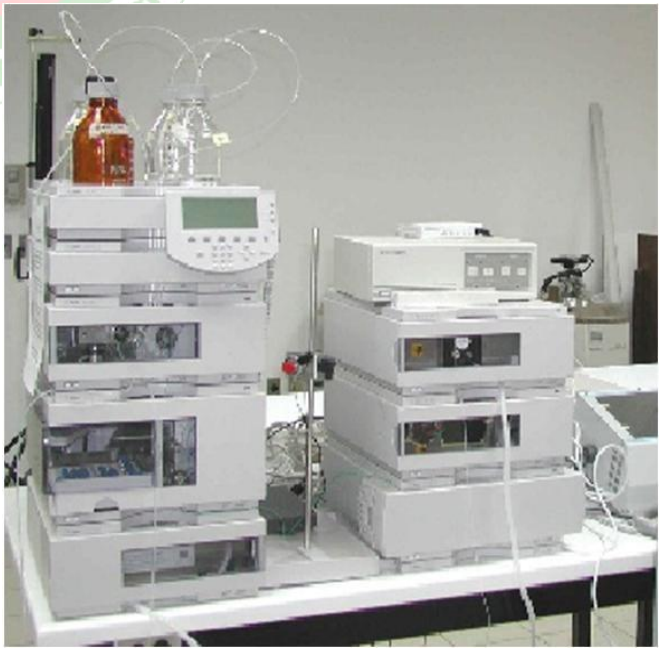


Figure: 1The of HPLC instrument

HPLC Method Development:[3]

Methods are developed for new products when no official methods are available. Alternate method for existing (Non-Pharmacopoeial) products are to reduce the cost and time for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants.7

Steps involved in Method development are. 6,7

- Understanding the Physicochemical properties of drug molecule.
- Selection of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation
- Method optimization
- Method validation (figure-2)

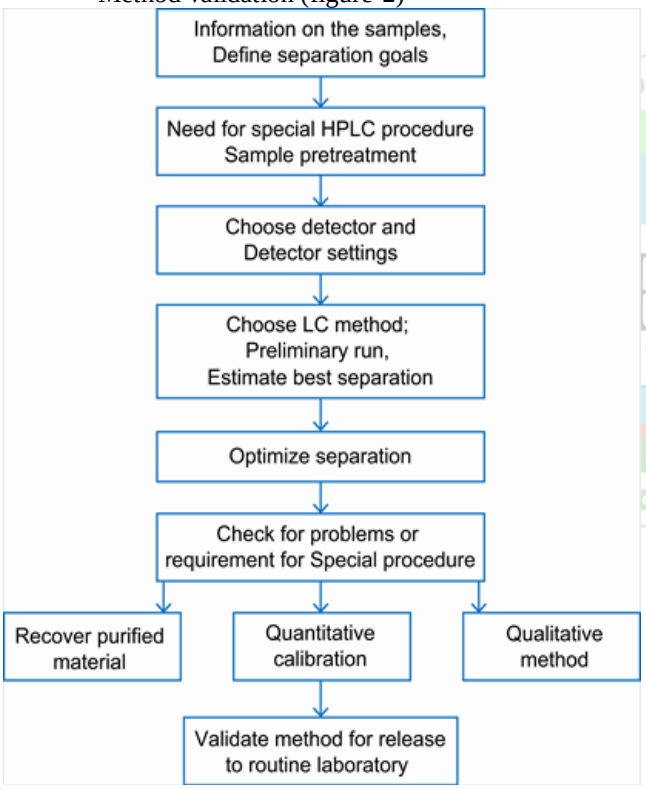


Figure 2: Steps involved in HPLC Method development

Method development and validation[4]

The International Organization for Standardisation (ISO) defines validation as the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled[21]. This definition primarily implies that a detailed investigation has been carried out and gives evidence that an analytical method, when correctly applied, produces results that are fit for purpose as well as it confirms the effectiveness of the analytical method with a high degree of accuracy. The importance of method validation has been emphasized since the late 40's when the American Chemical Society and Merck& Co., raised the issue of how mathematics and statistics are a necessary prerequisite for successful development and adaptation of new analytical methods. In addition, it was pointed out that statistical data analysis was a subject neglected by chemists developing experimental methods[22-25]. This paved the way for the implementation of method validation in analytical laboratories since the late 70's and the current worldwide recognition that method validation important component in any laboratory engaged in the development and establishment of standard methods. Now-a-days, there are several international renowed organizations offering guidelines on method validation and related topics.

The parameters involved in method validation are –

a) Precision
b) Accuracy
c) Limit of Detection
d) Limit of Quantification
e) Specificity
f) Linearity
g) Range
h) Robustness
i) System Suitability

It is important to understand the parameters or characteristics involved in the validation[26-29] process. The various performance parameters that are addressed in a validation process are as follows.

Precision: The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time while reproducibility involves precision study of different occasions, different laboratories, different batch of reagent, different analysts and different equipment.

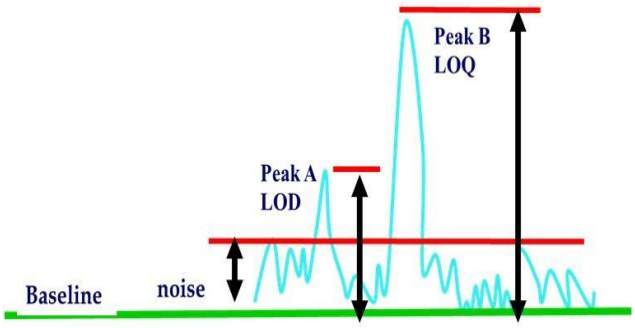
Accuracy: The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often be expressed as percent recovery by the assay of a known amount of analyte added. Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. Dosage form assays commonly provide accuracy within 3-5 % of the true value. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three

concentrations and three replications of each concentration).

Limit of detection: The limit of detection is the parameter of limit tests. It is the lowest level of analytes that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analytes concentration is above or below a certain level. The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analytes with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-tonoise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (S_a) which may be related to LOD and the slope of the calibration curve, 'b', by

$LOD = 3\ S_a/b$

Limit of quantitation: Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. The final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the limit of quantitation is approximately twice the limit of detection.



Selectivity/Specificity: The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. Selectivity may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analytes in the present expected levels of other components, compared the results obtained when the procedure is applied to the analytes in the presence of expected levels of other components, compared to the results obtained on the same analytes without added substances. When the other components are all known and available, selectivity may be determined by comparing the test results obtained on the analytes with and without the addition of the potentially interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of a standard addition of pure analytes to a material containing a constant level of the other components.

Linearity and range: The linearity of an analytical method is its ability to elicit test results that are directly (or by a well-defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analytes. The linear range of detectability that obeys Beer's law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation $y = ax + b$ together with the correlation coefficient of determination (r). For the method to be linear the r value should be close to 1. The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written[5].

Ruggedness: The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method. For the determination of ruggedness, the degree of reproducibility of test result is determined as function of the assay variable. This reproducibility may be compared to the precision of the assay under normal condition to obtain a measure of the ruggedness of the analytical method.

Robustness: The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an

indication of its reliability during normal usage. The determination of robustness requires that method's characteristics are assessed when one or more operating parameter varied.

Stability and system suitability tests: Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 hour stability is desired for solutions and reagents that need to be prepared for each analysis. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test is a method that is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. The nature of the test and the acceptance criteria will be based upon data generated during method development optimization and validation experiments.

System Suitability Testing: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated[6].

The parameters that are affected by the changes in chromatographic conditions are:

a) Retention (K_A)
b) Resolution (R_S)
c) Capacity factor (k′)
d) Selectivity (□)
e) Column efficiency (N) and vi)Peak asymmetry factor (A_S)

Retention (K_A) : The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

KA =VA – V0 /V0=tA- t0 /t0

Where,

V_A = Elution volume of A

V_0 = Elution volume of a non - retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes.

Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times(tA- t0 /tB- t0)is better when data need to be transferred between different chromatographs.

Resolution (R_S): The resolution, R_s of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_S is 1.5. It is calculated by using the formula,

$$R_f = \frac{Rt^2 - Rt^1}{0.5\,(W_1 + W)_2}$$ Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2 W_1 and W_2 are peak widths of components 1 and 2.

Capacity factor (k'): Capacity factor, k′ is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an isocratic separation. The ideal value of k′ ranges from 2-10. Capacity factor can be determined by using the formula,

k ' =V1 -V0/V_0

Where, V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The value of k′ of individual bands increase or decrease with a change in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water/organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k′ of the bands by a factor of 2-3.

Selectivity (α):

The selectivity (or separation factor), α is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$_A$ =V 2 - V 0 /V 1 - V 0

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak respectively.[7]

Column efficiency:

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16 \left(\frac{Rt^2}{w}\right)_2$$

Where, R_t is the retention time and w is the peak width.

The column plate number increases with several factors:

1. Well-packed columns (column 'quality')
2. Longer columns
3. Lower flow rates (but not too low)
4. Smaller column-packing particles
5. Lower mobile-phase viscosity and higher temperature
6. Smaller sample molecules.

Peak asymmetry factor (A_S):[8]

Peak asymmetry factor, A_S can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

As = b/a

CONCLUSION

In recent years development of the analytical methods for identification, purity evaluation and quantification of drugs has received a great deal of attention in the field of pharmaceutical analysis. This review describes HPLC method development and validation in general way. A general and very simple approach for the HPLC method development for the separation of compounds was discussed. Knowledge of the physiochemical properties of the primary compound is of utmost importance prior to the any HPLC method development. The selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

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