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# "RP-HPLC: A Versatile Analytical Technique for Pharmaceutical Analysis - A Review"

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## ABSTRACT:

High Performance Liquid Chromatography (HPLC) is a key analytical method widely used in in pharmaceutical research for the separation, identification, and measuring of drug substances and related impurities. Owing to its high resolution, sensitivity, and reproducibility, HPLC—particularly reverse-phase HPLC—is the preferred method of choice for routine quality control and stability studies. This review presents a systematic overview of HPLC principles, instrumentation, and chromatographic modes, with emphasis on method development strategies and validation requirements. Key factors influencing method development, such as mobile phase composition, column selection, detection parameters, and sample preparation, are discussed in detail. The article also outlines method validation parameters as recommended by FDA and ICH guidelines, including specificity, accuracy, precision, linearity, robustness, ruggedness, and limits of detection and quantification. In addition, the importance of stability-indicating methods is highlighted through a discussion of forced degradation tests under acidic, basic, oxidative, thermal, photolytic, and humidity stress conditions. This review aims to provide a concise and regulatory-compliant framework for the development and validation of robust HPLC methods in pharmaceutical analysis.

**Key words:** RP-HPLC, method development, validation, stability indication.

## INTRODUCTION:

Analytical chemistry is a branch of chemistry that deal with the identification of components and the determination of the number of components of elements or samples or mixtures.[1] In analytical chemistry, chromatographic techniques are most widely used for effective separation and purification of compounds in pharmaceuticals.[2] The chromatography derived from the Greek words, chromo meaning 'colour' and, graphs meaning to 'write'. In modern chemistry, chromatographic techniques are used for qualitative and quantitative analysis.[3] High-performance liquid chromatography (HPLC) is the most effective technique in identifying, measuring, and separating components based on their affinities towards the stationary and mobile phase at high pressure. In HPLC the components which have lower affinity towards the stationary phase elute fast, and the components which have higher affinity towards the stationary phase elute slowly. HPLC has several advantages: high resolution, high sensitivity, good repeatability and small sample size.[4]

**Types of HPLC:** [5-11]

High performance liquid chromatography has two types; Normal phase HPLC (NP-HPLC) and reverse phase HPLC (RP-HPLC).

1. **Normal phase HPLC**:

In normal phase chromatography, the stationary phase is polar and the mobile phase is nonpolar. The analytes that are polar have enhanced adsorption capacity towards the stationary phase and slowly elute. The analyte which are non-polar have less adsorption capacity towards stationary phase and elute fast. This technique is rarely used.

2. **Reverse phase HPLC**:

In reverse phase chromatography the stationary phase is nonpolar and the mobile phase is polar. The analyte which are polar have less adsorption capacity towards the stationary phase and elute fast. The analytes that are nonpolar have enhanced adsorption capacity towards stationary phase and slowly elute. There are two types of elution: isocratic and gradient elution. The elution of a simple mixture with the a same polarities is isocratic elution. The elution of complex mixture with different polarities is gradient elution. The principle of RP-HPLC is based on the hydrophobic interaction. This technique is most commonly used. The column used for RP-HPLC are BDS Hypersil C18 column (250 × 4.6 mm, 5µm), Agilent 5 HC C18-(150 × 4.6mm, 5µm). The parameters used in RP-HPLC are retention time, retention volume, separation factor, resolution, theoretical plate, HETP- height equivalent to the original plate, efficiency (no of theoretical plates) and asymmetry factor- fronting or tailing.

**Instrumentation of High-performance liquid chromatography (HPLC)**:[12]

In reverse phase chromatography, the stationary phase is non-polar and the mobile phase is polar. Polar analytes have less attraction to the stationary phase and move through the column quickly. Non-polar analytes are more strongly retained and elute more slowly. There are two types of elution: isocratic and gradient. Isocratic elution is used for simple mixtures with similar polarities, while gradient elution is used for complex mixtures with different polarities. RP-HPLC works on the principle of hydrophobic interactions and is widely used. Common columns for RP-HPLC include the BDS Hypersil C18 (250 x 4.6 mm, 5µm) and Agilent 5 HC C18 (150 x 4.6 mm, 5µm). Key parameters in RP-HPLC are retention time, retention volume, separation factor, resolution, theoretical plates, HETP (height equivalent to a theoretical plate), efficiency (number of theoretical plates), and asymmetry factor (fronting and tailing).

**Components of HPLC:** [13-15]

i. Infusion pump:

A steady motion of the mobile phase through the HPLC column depends on the infusion pump, also known as the solvent delivery system. High pressures, usually up to 6,000 psi (400 bar) for regular HPLC and up to 18,000 psi (1,200 bar) for Ultra High-Performance Liquid Chromatography systems, can be produced by contemporary HPLC pumps. These pumps can run in two different modes: gradient mode, which mixes many solvents to reach varied concentrations throughout the run, and isocratic mode, which uses a single solvent. Due to its dependability in producing high pressure, piston pumps are the supremely used kind in HPLC; however, unless they are muted by additional components, they may cause pulsations that could impair performance.

ii. Sample injector:

An HPLC injector may be operated manually or through an automated system. It must deliver sample volumes typically between 0.1 and 100ml with excellent reproducibility, while also tolerating the high pressure of the system, which can reach up to 4000psi.

Types of injector:

I. Manual injection
II. Automatic injection
III. Septum injector
IV. Stop flow injector
V. Rheodyne injector

iii. Chromatographic Column:

The separation of chemicals takes place in the column. It is loaded with a stationary phase, which is usually made up of tiny porous silica particles that interact differently with different sample components as they move through. Achieving the best possible separation efficiency depends on the stationary phase's chemistry and design. To sustain high pressures, columns are typically enclosed in stainless steel. They come in a variety of sizes and packing materials designed for certain uses.

Types of column:

I. Guard column: A guard column is set before the analytical column to protect it and extend its lifespan. The guard column is packed with material similar to that of the analytical column, although the particles are generally huge.
II. Analytical columns: Analytical columns are the fundamental component of an HPLC system. These liquid chromatography columns usually range from 10 to 30 cm in length and are typically straight. The most commonly used column is 25 cm long, has a 4.6 mm internal diameter, and is packed with 5 µm particles, providing approximately 40,000–60,000 theoretical plates per meter.

iv. Detector:

In HPLC, the detector is placed at the end of the column and identifies the analytes as they exit the chromatographic system. The detector is responsible for sensing the individual molecules that elute from the column and measuring their quantity so the chemist can perform a quantitative analysis of the sample.

The detector used in RP HPLC are;

i. UV visible detector
ii. Charged Aerosol Detectors
iii. Mass spectroscopic detector
iv. Fluorescence Detectors
v. Refractive index detector
vi. Electrical conductivity detector

5. Data Recording and Processing System:

Electronic integrators can range from simple to advanced, and they differ in how well they can handle, store, and reanalyse chromatographic data. The computer takes the detector's signal for each substance and turns it into a chromatogram that is easy to read. Often called the data system, the computer not only operates all parts of the HPLC instrument but also uses the detector signal to find out when each component comes out of the column (its run time), which helps identify the sample's components.

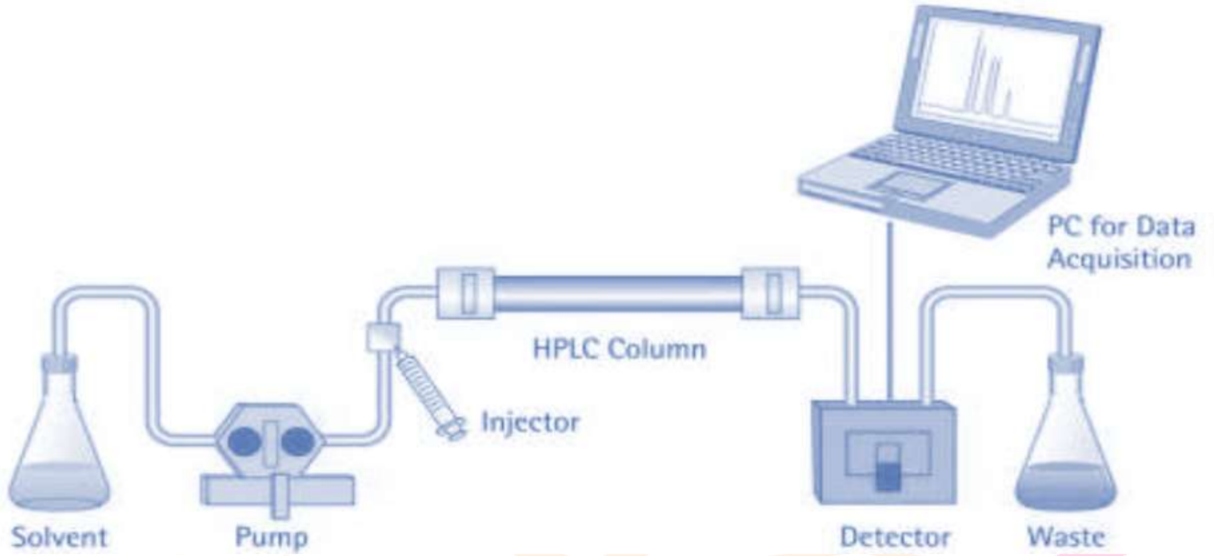


**Fig 1. HPLC[7]**

## METHOD DEVELOPMENT BY HPLC: [2,16-21]

1. **Selection of the HPLC method and initial system:**

When generating an HPLC method, the first thing to do is check the existing literature to see whether the separation has already been done and under what conditions. This helps avoid wasting time on pointless experiments. When choosing an HPLC setup, it should be suitable for analysing the sample. For instance, if the sample contains polar compounds, reverse-phase HPLC is usually the better choice because it provides good retention and separation, while normal-phase HPLC would be far less practical.

2. **Choice of Mobile Phase:**

Mobile phase is a key part of developing an HPLC method. It acts as the solvent that moves the sample through the column, and its composition has a major effect on how well the analytes are separated.

**3. Choosing Chromatographic Conditions:**

At the start of method development, the detector, column, and mobile phase are selected to create the first "scouting" chromatograms of the sample. Most often, a reversed-phase setup with a C18 column and UV detection is used. At this stage, the analyst decides whether to use a gradient method or an isocratic method, with each option having its own advantages depending on the separation goals and the properties of the analytes.

**4. Column Selection:**

Choosing the right column is also a critical step in HPLC method development. Columns differ in their size, packing material, and molecule diameter, and these factors influence how well the separation performs. In reverse-phase HPLC, C18 silica gel is the most widely used stationary phase because it offers strong retention for many types of compounds. However, other options like C8 or phenyl columns may work better for certain specific analyses.

**5. Optimization of mobile phase:**

Optimization of mobile phase involves the:

i. Buffer selection
ii. Effect of $p^H$
iii. Selection of wavelength
iv. Effect of organic modifier

**6. Sample preparation:**

The sample should preferably be dissolved in the starting mobile phase. If this cannot be done because of solubility or stability issues, small amounts of formic acid, acetic acid, or salts can be added to help it dissolve. These additives typically do not affect the separation, as long as the sample volume injected is small compared to the column volume.

**7. Method optimization:**

After obtaining acceptable initial separations, the method conditions are optimized—by systematically adjusting factors like pH, mobile-phase composition and ratio, gradient, flow rate, temperature, sample amount, injection volume, and diluent—to achieve the required separation and sensitivity for a stability-indicating assay.

8. **VALIDATION:** [22]

The FDA defines validation as a process used in drug production and control to ensure that drug products are correct, strong, good quality, and pure. According to FDA guidelines from May 1987, a validation package must give all the needed information and test steps to show that a system or process meets its set standards. Validation is the act of proving that any process, equipment, material, activity, or system actually works as planned. This idea started in the United States in 1978. Over the years, validation has grown to cover many areas, like testing methods for medicine quality, computer systems used in clinical studies, and process control. Method validation is about checking whether an analytical method is right for its purpose. It makes sure the method is clear, works well, gives reliable results, is accurate, and can detect small amounts. A big part of validation involves using equipment that is correct, well-calibrated, and works properly. Validation ensures all analytical methods are checked thoroughly and either approved for use or rejected if they don't meet the required standards.

The FDA, USP, and ICH commonly recommend the following parameters; [23-29]

i. System suitability
ii. Solution stability
iii. Specificity

iv. Accuracy
v. precision
vi. Linearity
vii. Range
viii. Ruggedness
ix. Robustness
x. LOD and LOQ

1. **System Suitability**:

System suitability testing (SST) is a crucial component of most analytical processes. System suitability tests give extra surety that, at the time of analysis, the method is producing results that are both accurate and precise. System suitability tests are usually carried out before analysing real samples. They use standard solutions with known analyte concentrations, and the method's performance is assessed by comparing key metrics with preset acceptance criteria defined during validation.

Regular pre-analysis checks include ensuring a tailing factor of ≤2, theoretical plates of ≥2000, an RSD of ≤2%, and a resolution of ≥2 for critical peak pairs.

2. **Solution stability**:

During validation, the stability of standards and samples is evaluated under different conditions, such as routine laboratory environments, recommended storage settings, and occasionally inside the instrument itself. Understanding the effects of different storage conditions is vital for preserving the accuracy and integrity of analytical results over time. This insight helps determine the proper handling and storage procedures for the substances involved in the analysis.

3. **Specificity:**

The ability to determine the degree of specificity of the target analyte in the presence of other expected components. The specificity of a chromatographic method is evaluated by demonstrating that the analyte can be distinctly separated from other possible components like impurities, degradation products, or excipients**.** This is especially important in complex matrices, such as biological fluids or pharmaceutical formulations, where numerous components can coexist.

4. **Accuracy:**

Accuracy is the closeness of a measured value to the true or accepted value**.** Accuracy is calculated as the percentage of analyte recovered in the assay from the test results. It is typically expressed as the recovery of well-known added analyte, indicating how closely the analytical method reflects the true values.

$$\text{Recovery} = (\text{Measured Value} / \text{True Value})$$

For most analytical methods, an accuracy between 98% and 102% is generally deemed acceptable.

- Determination methods:

i. Spiked – placebo recovery method
ii. Standard addition method
iii. Recommended Data
iv. Acceptance criteria

v. Using an analytical technique on an analyte with a known concentration

**5. Precision:**

In analytical methods, precision reflects how closely a sequence of measurements agree when taken under defined conditions from multiple samples of the same homogeneous material. It is a key parameter for evaluating the reproducibility of the entire analytical process.

Precision is expressed as SD or RSD

$$\%RSD = \frac{STANDARD\ DEVIATION}{MEAN} \times 100$$

- Types of precision:

i. Interday precision: It is analytical procedure carried out on different day by different analyte.

ii. Intraday precision: It is analytical procedure carried out multiple times on same day by different analyte.

**6. Linearity:**

Linearity is typically evaluated using the confidence interval of the regression line's slope. The ICH recommends testing at least five different concentrations to establish linearity. Linearity should be evaluated by visually inspecting a plot of the signal versus analyte content or concentration

**7. Range:**

The analytical method's range is defined as the span between the highest and lowest concentrations that can be measured with demonstrated precision, accuracy, and linearity. The range is usually reported in the same units as the analytical results, such as percentage or parts per million.

**8. Ruggedness:**

The extent to which test results remain consistent and repeatable when the same sample is analysed under standard testing conditions.

The degree to which test results can be consistently reproduced when analyzing the same sample under various normal test conditions.

- Instruments
- Days
- Analyst
- Reagent
- Columns
- TLC plate

**9. Robustness:**

Robustness of an analytical method refers to its reliability under the normal conditions and its capacity to tolerate small, deliberate changes in method parameters. Robust methods are less affected by changes or variations, enhancing their reliability in practical analytical applications.

**10. LOD and LOQ:**

The limit of detection (LOD) of an analytical method is the smallest amount of analyte that can be detected in a sample, though it may not be quantified precisely. There are various approaches to determining the LOD.

$$\text{LOD} = \frac{3.3\sigma}{S}$$

Where,

σ = Standard deviation of Intercepts

S= Mean of slopes of the calibration

The limit of quantitation (LOQ) for an analytical method is the smallest amount of analyte in a sample that can be measured both precisely and accurately. It serves as a key parameter for evaluating low analyte levels in test matrices.

$$\text{LOQ} = \frac{10\sigma}{S}$$

LOD and LOQ are typically determined by statistically analysing calibration curves or testing blank samples spiked with low analyte concentrations. A common method involves using signal-to-noise ratios, with LOD estimated at S/N = 3:1 and LOQ at S/N = 10:1.

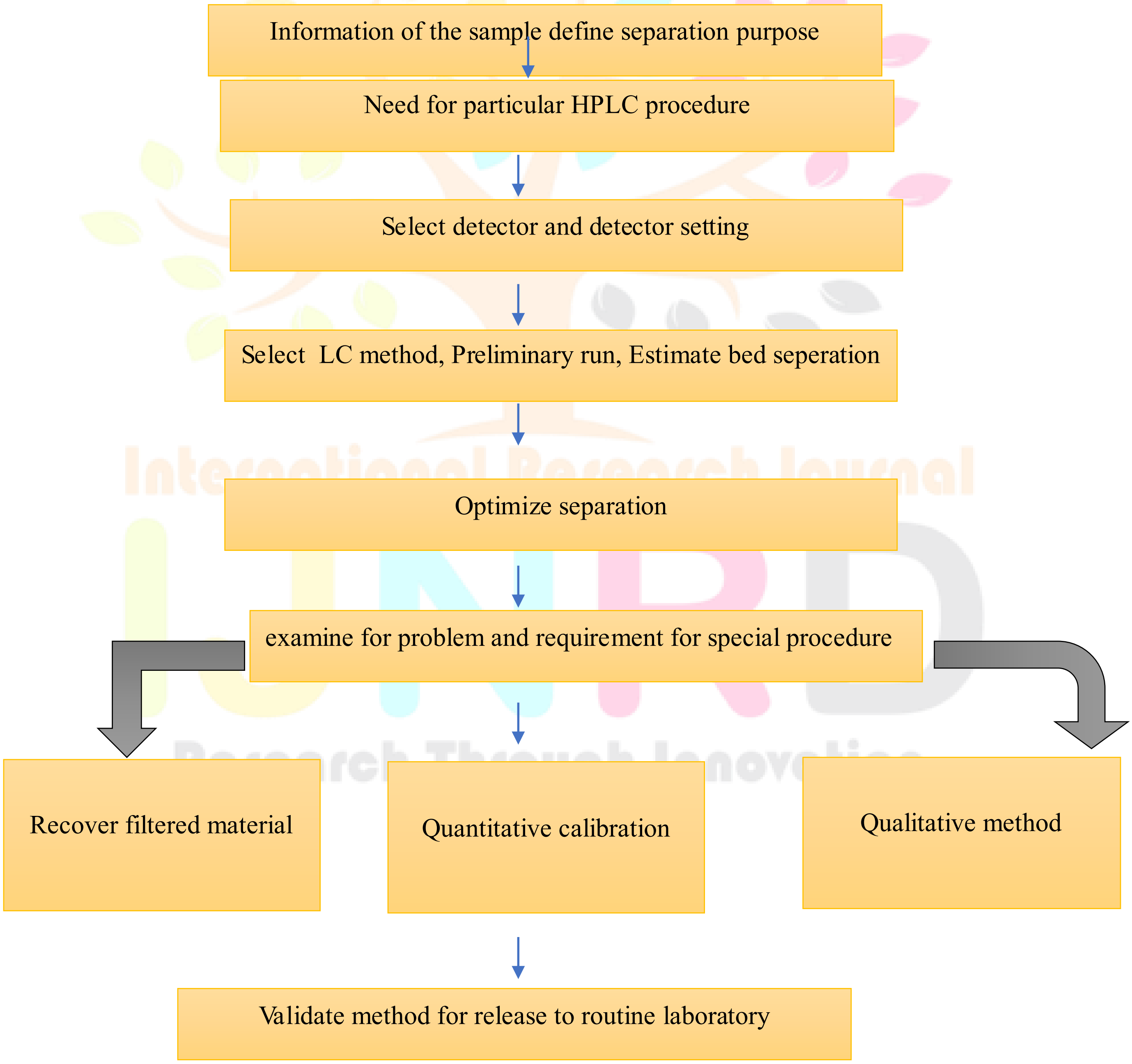


**Fig.2 flow chart of method development.** [7]

- **Stability indicating parameter**: [30-32]

According to food and drug administration guidance, a stability-indicating test procedure is a validated quantitative analytical method that is capable of identifying changes in the quality attributes of a drug substance or drug product over the course of storage. Stability programs are conducted to initiate suitable storage conditions and to determine the expiration date.

**i. Acid hydrolysis:**

Hydrochloric acid or sulfuric acid is commonly employed for acid hydrolysis. In this reaction, the compound breaks down through interaction with water. It is the process of inducing acid-catalysed degradation by treatment with a dilute acid, such as 0.1 N hydrochloric acid.

**ii. Basic hydrolysis:**

sodium hydroxide or potassium hydroxide is typically used for base hydrolysis. Alkaline degradation is simulated by exposure to a dilute base, such as 0.1 N sodium hydroxide.

**iii. Oxidative degradation:**

Oxidative stability is assessed by exposing the material to oxidizing conditions, for example using hydrogen peroxide at a concentration of about 3% $H_2O_2$. In some instances, even exposure to high concentrations of hydrogen peroxide under severe conditions does not result in significant degradation.

**iv. Thermal degradation:**

Thermal degradation testing should be conducted on both the active pharmaceutical ingredient and the dosage form, with or without humidity. Solid drug substances, placebos, and drug product samples should be subjected to heat in the presence and absence of humidity, while liquid drug substances and drug products can be tested under heat without humidity.

There are two types of heat;

a. Dry heat

b. Wet heat

**v. Photolytic degradation:**

sunlight: Photolytic studies should involve exposing the drug solution to sunlight. The solution must be subjected to sunlight for a duration of four days.

**vi. Humidity stress:**

Humidity significantly influences the formation of potential degradation products in both the active pharmaceutical ingredient and the finished dosage form. Typically, exposure to 90% relative humidity for period of one week is recommended to generate forced degradation samples

## CONCLUSION:

HPLC remains an essential and reliable analytical technique in the pharmaceutical industry due to its versatility and analytical performance. The development of an effective HPLC method requires a structured approach involving careful optimization of chromatographic parameters to achieve adequate resolution and reproducibility. Method validation, conducted in accordance with FDA and ICH guidelines, confirms the suitability of the analytical procedure for its intended application and ensures consistent data quality.

Stability-indicating HPLC methods play a vital role in understanding drug degradation pathways and ensuring product quality throughout its shelf life. In conclusion, the integration of systematic method development, comprehensive validation, and stability studies is fundamental for regulatory compliance and effective pharmaceutical quality control. This review provides a scientific foundation for researchers and analysts involved in HPLC-based method development and validation.

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