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Method Development by High-Performance Liquid Chromatography: Review

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ABSTRACT

In pharmacological, biological, toxicological and environmental investigations, estimating trace amounts of compounds is critical. Because even a minuscule amount of a material can be harmful or toxic. Among the several chromatographic processes, one of the most effective analytical procedures is HPLC. HPLC is the most flexible, safest, and quickest chromatographic method for quality control of active pharmaceutical compounds. The fundamental advantage of the proposed technique is that different pharmaceutical ingredients may be determined with just minor modifications in detection wavelength and mobile phase composition by utilizing a single chromatographic apparatus. The current study focuses on important information such as the development of HPLC method, their validation as per the ICH guidelines, and the estimation of diverse drug combinations in pharmaceutical dosage forms.

INTRODUCTION:

At the turn of the twentieth century, Russian-Italian botanist Mikhail Semyonovich Tswett discovered chromatography. The Greek word chromatography means "color writing", He saw a colorful visual of his first plant pigment separation, which is a physicochemical procedure based on the phenomena of adsorption that he used to separate composite mixtures. R. L. M. Synge and A. J. P. Martin devised partition chromatography in 1941 at Cambridge University in the United Kingdom. In 1952, they were given a noble prize for their efforts. The value of Chromatography is expanding quickly in pharmaceutical analysis. Chromatography enables the separation, identification, and measurement of structurally related compounds with pinpoint accuracy. The purity assessment of finished products and intermediates is another key application of chromatographic methods (detection of decomposition products and by-products). As a result of the foregoing, chromatographic methods are increasingly being included in the most recent editions of pharmacopeias and other testing standards. High-performance, high-pressure, high-resolution, and high-speed liquid chromatographies are all terms used to describe the modern form of column chromatography. [1, 8]

Columns packed with small glass beads with porous layers on their surface, developed significant resistance to the liquid flow and the Prof. Horwath was forced to build an instrument that allowed the development of the continuous flow of liquid through this column. This was the origin of high-performance liquid chromatography. HPLC is abbreviated as high-pressure liquid chromatography or high-performance liquid chromatography. High-performance liquid chromatography is an important analytical method for assessing pharmaceutical compounds. It's been used before to address half of all pharmaceutical analytical problems. It can detect and quantify various medicines and drug-related degradants that occur during storage or production, as well as any drugs and drug-related impurities introduced during synthesis. HPLC is an advanced technique of column liquid chromatography that pumps solvent/mobile phase at adequately high pressure and carry analyte or sample combination through column containing chromatographic packing material/ stationary phase which may be solid or liquid. [1, 2, 3]

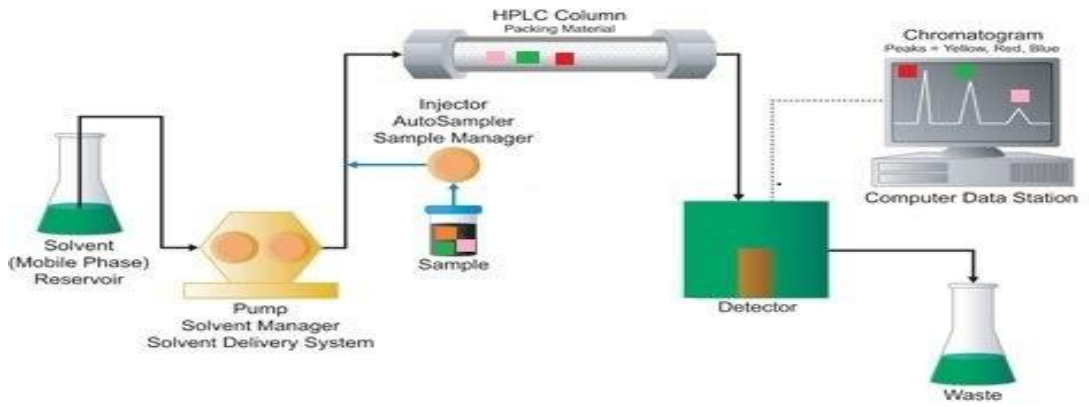


Figure 1: Instrumentation of HPLC

There are generally five different types of HPLC based on the phase system used in the process: [4, 5, 6, 8, and 9]

1) Normal phase chromatography (NP-HPLC): This approach is used to separate analytes according to their polarity. Polar stationary phase and nonpolar mobile phase is used hence elution time increases with increased analyte polarity.

2) Chromatography in the reversed-phase (RP-HPLC): This method follows the hydrophobic interaction principle and uses non-polar stationary phase and moderately polar mobile phase. In today's HPLC, reversed-phase chromatography is the most often used separation method. Over 65 percent (perhaps up to 90 percent) of all HPLC separations are performed in reversed-phase mode, according to estimates. The ease, adaptability, and the reversed-phase approach's scope, which can handle compounds of various polarity and molecular mass, are among the reasons for this.

3) Size exclusion chromatography (SEC): Also called as gel permeation or gel filtration chromatography. Separates particles based on size.

4) Ion exchange chromatography: The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength.

5) Bio-affinity chromatography: Based on specific reversible interaction of proteins with ligands.

Method development and validation:

There are many factors to consider when developing analytical methods. Initially collect information about the physicochemical parameters of the analyte (pKa, log P, and solubility) are then used to decide which technique of detection is best for analysis. Method development entails several steps which are:

- Drug's physicochemical qualities
- Selection of chromatographic conditions
- Sample preparation
- Method optimization
- Validation of the developed method

Physicochemical properties of analyte: [6]

The physicochemical qualities of the analyte are crucial in the development of methods. For development, physical properties of analytes such as solubility, polarity, pKa, and pH must be investigated. Polarity aids analysts in determining the solvent and mobile phase composition. The concept of polarity can be used to explain solubility. Polar and nonpolar solvents, such as water and benzene, do not mix. Hence only like dissolves like. The mobile phase is chosen based on the analyte's solubility. pH is defined as the negative of the logarithm of the hydrogen ion concentration to base 10. pH= -log1[H_3O]. Choosing the right pH results in symmetrical and sharp peaks. The pKa value is a property of a substance that indicates how easily it gives off proton.

Selection of chromatographic conditions:

Buffer selection: [6]

The buffer is chosen based on the desired pH. The pH range for reversed-phase chromatography is 2 to 8.

When choosing a buffer, there are a few things to keep in mind which are as follows:

1. Phosphate is more soluble in methanol/water.

2. Salt buffers are hygroscopic, and phosphorus is more soluble in methanol/water. Some Changes in chromatography may result as a result of this (increased tailing of basic compounds, and possibly selectivity differences).

3. TFA degrades over time, is volatile, and absorbs UV light at low wavelengths.

4. Microbial development has the potential to degrade chromatographic performance.

5. Phosphate buffer increases silica dissolution and significantly at pH 7, it lowers the lifespan of silica-based HPLC columns.

6. Ammonium bicarbonate buffers are only stable for 24 to 48 hours in most cases.

7. A 0.2-m filter should be used to filter the buffers.

8. The mobile phase must be degassed.

For small compounds, a buffer it is adequate to have a concentration of 10-50 mM; However, no buffer with more than 50% organic content should be used. It is determined by the buffer type and concentration. The most frequent buffer systems for reversed-phase HPLC are phosphoric acid and its sodium or potassium salts. Because buffers regulate pH best at its pKa, the buffer's pKa must be close to the intended pH. Choosing a buffer with a pKa value less than 2 units of the desired mobile phase pH is the usual norm.

HPLC Detectors: [4, 6, 7]

The detector's job in HPLC is to track the mobile phase as it exits the column and emits a response before signalling a peak on the chromatogram. There are two types of HPLC detectors: bulk property detectors and solute property detectors.

1) Bulk property detectors: These detectors work by differentially measuring a property shared by both the sample and the mobile phase. Detectors that measure the refractive index, conductivity, and dielectric constant are examples of such detectors.

2) Solute property detectors: These detectors respond to a physical feature of the solute that is absent from the pure mobile phase. With or without the mobile phase removed before detection, these detectors monitor a sample-specific property. The mobile phase must be removed before detection for the moving wire flame ionization detector and the electron capture detector, however, the spectrophotometric (UV or UV-Vis) detector, fluorescence detectors, polarographic, electro-chemical, and radioactivity detectors do not.

Because many solvents used in HPLC do not absorb to any considerable level, UV-Vis and fluorescent detectors are ideal for gradient elution. The UV detector (fixed and variable wavelength), the electrical conductivity detector, the fluorescence detector, and the refractive index detector are the four most common detectors used in LC analysis. Detectors like this are used in approximately 95% of all LC analytical applications. The detector to use is determined by the sample and the goal of the analysis.

Column selection: [6]

The heart of the system is the column. The type of column used for a particular separation is determined by the compound and the purpose of the analysis. Silica, polymers, and alumina are some of the matrices used to sustain the stationary phase. Silica is the most often used component in the production of packaging materials (SiO_2, H_2O). The nature, shape, and particle size of silica aid in the separation. The presence of silanol groups on silica's surface gives it polarity, which is useful in adsorption chromatography with non-polar organic eluents. Silica can be significantly transformed by combining with organochloro silanes or organoalkoxy silanes, resulting in Si-O-Si-R surface connections. The hydrocarbon modification binds to silica, resulting in a non-polar surface that may be used for reversed-phase chromatography with water and organic solvents. The most prevalent material is octadecyl silica, which has a C18 chain. The bonding to silica of other organic compounds including groups such as phenyl, nitro, amino, and hydroxyl results in a wide spectrum of solids with intermediate surface polarity. Strong ion exchangers with sulphonic acid or quaternary ammonium groups bound to silica are also available. The pH range for columns is 2 to 8 because siloxane linkages are broken at pH-2 and silica can dissolve at pH levels above 8. Capacity factor, selectivity, efficiency, and elution are all influenced by the nature of the stationary phase.

Column temperature control:

Because temperature can alter selectivity, column temperature control is critical for long-term technique repeatability. For good reproducibility, a target temperature of 30-40^{0}C is usually sufficient. It is recommended that the column be thermally stated to control the temperature during method development and validation to avoid possible temperature swings.

Mobile phase: [4, 6]

The eluting power of a sample is influenced by the overall polarity of the mobile phase, the polarity of the stationary phase, and the composition of the sample components. As the polarity of the solvent rises, so does the eluting power in normal phase separations, but decreases as the polarity of the solvent increases in reversed-phase separations. To obtain ideal separation conditions, two solvents together might be utilised. Other solvent criteria to consider for a successful separation include boiling point, viscosity, detector compatibility, flammability, and toxicity. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. In RP-HPLC, the solvents acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) are widely utilised. The mobile phase and gradient parameters are chosen based on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture. The aqueous buffer performs a variety of functions. The mobile phase protonates free silanols on the column and decreases peak tailing when the pH is low. In contrast, neutral & basic molecules will be held more at higher pH levels, while ionised acidic compounds will elute sooner. Peak splitting can occur when the pKa of a chemical is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The optimum starting choice for the mobile phase during the technique is a mixture of acetonitrile and water.

Separation techniques**:**

I. Isocratic separations: constant eluent composition. Number of compounds which could be resolved is not very high.

II. Gradient separation: varied eluent composition employed for complex multi-component samples.

Selection of isocratic or gradient mode depends on the number of active components to be resolved. To decide, initial gradient run is performed and ratio between the first and last components is calculated. When the determined ratio is less than 0.25, isocratic is sufficient; when the ratio is more than 0.25, gradient would be advantageous.

Preprocessing of samples for technique development: [5, 6]

Sample preparation is a crucial stage in the development of a method. The purpose of sample preparations is to create a processed sample that leads to better analytical results compared

with the initial sample. The drug substance under investigation must be stable in solution (diluent). Initially preparation of solutions in amber flasks should be performed. The sample should be filtered (0.22 or 0.45micrometer filter generally recommended).

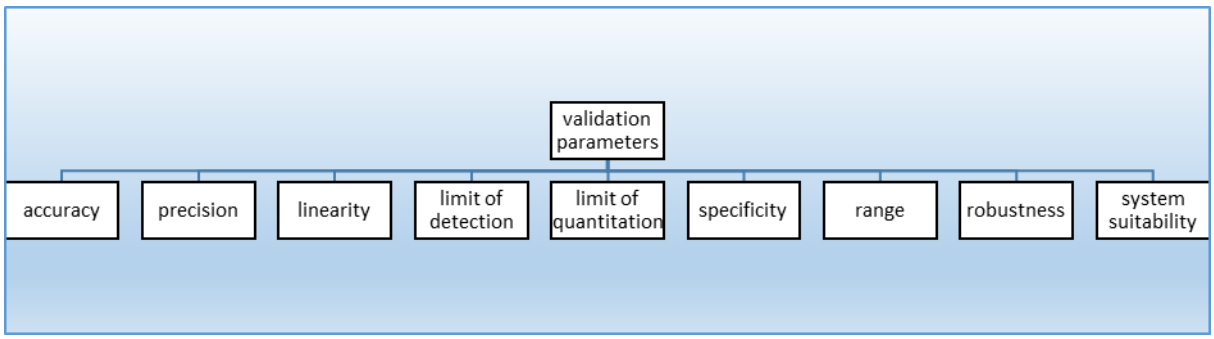
Method optimization:

To get the necessary separations and sensitivity, the experimental conditions should be tuned. Planned/systematic study of factors such as pH (if ionic), mobile phase components and ratios, gradient, flow rate, temperature, sample amount, injection volume, and diluents solvent type will be used to produce experimental conditions.

Method validation:

It is a technique for determining whether a procedure's performance characteristics match the requirements for its intended usage. Analytical methods are validated following ICH criteria. Prior to usage, a written and authorized procedure for method validation is required. The reliability and quality of analytical results can be checked or inspected through method validation. The method validation procedure requires the use of equipment that is within specification, operation, and properly calibrated. Validation or revalidation of analytical procedures is required. [4, 11]

Validation parameters: [4, 6]



1. **Accuracy** is the degree to which a measured value resembles a genuine or accepted value. The difference between the mean value found and the genuine value is called accuracy. To confirm that there is no interference, these should be compared to standard and blank solutions. After that, the accuracy is calculated as a percentage of the analyte recovered by the assay based on the test results. The recovery of known, added amounts of analyte by test is a common expression.

2. **Precision:** closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision may be considered at three levels such as repeatability, intermediate precision and reproducibility.

- Repeatability: It is the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

- Intermediate precision: It is carried out with differences within laboratories, such as different analyzers, different equipment, various days, and so on.

- Reproducibility: It is performed between laboratories for collaborative studies.

3. **Linearity:** is the capacity of an analytical technique to provide a response that is proportionate to the analyte concentration in a sample. The confidence limit around the regression line's slope is commonly used to express linearity. The ICH recommendation recommends a minimum of five concentrations for establishing linearity. R^2 >0.995.

4. **Detection limit:** An individual procedure's detection limit (DL) or limit of detection (LOD) is the smallest quantity of analyte in a sample that can be identified but not always measured as a correct value. A signal-to-noise (S/N) ratio can be used to determine the LOD (3:1) in analytical processes with baseline noise, which is commonly stated as the analyte concentration in the sample. The signal-to-noise ratio is calculated as follows: s = H/h. Where H is the height of the component's corresponding peak. h is the absolute magnitude of the biggest noise fluctuation from the baseline of a blank solution's chromatogram. LOD= 3.3s Where s-Average of slope and -Standard deviation.

5. **Quantitation limit:** The lowest amount of analyte in a sample that can be quantitatively quantified with sufficient precision and accuracy is known as the limit of quantitation (LOQ) or quantitation limit of an analytical process. The LOQ is commonly calculated from a determination of the S/N ratio (10:1) and validated by injecting standards that yield this S/N ratio and have a percent relative standard deviation that is acceptable. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is usually estimated from a determination of the S/N ratio (10:1) and is usually confirmed by injecting standards that give this S/N ratio and have an acceptable percent relative standard deviation as well. LOQ= ten seconds Where s-Average of slope and -Standard deviation.

6. **Specificity:** is the capacity to assess the analyte definitively in the presence of potential contaminants such as impurities, degradation products, and excipients.

7. **Range:** is defined as the range of analyte concentrations in a sample for which it has been established that the analytical technique has a satisfactory level of precision, accuracy, and linearity.

8. **Robustness:** is a measure of an analytical technique's capacity to stay unaffected by modest but deliberate alterations in method parameters (e.g., pH, mobile phase composition, temperature, and instrumental settings) and provides an indicator of its reliability during routine use.

9. **System suitability parameters:** Pharmaceutical businesses undertake system appropriateness assessments to see if a chromatographic system will be used regularly in pharmaceutical laboratories. The following are the system appropriateness parameters that were investigated:

I. Resolution: The ratio of the distance between two peak maxima is defined as Rs of two nearby peaks. It's the ratio of the difference between two solutes' retention durations to their average peak width. The optimal value of Rs for baseline separation is 1.5.

II. Capacity factor: is the ratio of the number of solute molecules in the stationary phase to the number of solute molecules in the mobile phase. It's a metric for how well a sample molecule is held by a column during isocratic separation. The ideal value of capacity factor k is between 2 to 10.

III. The number of theoretical plates/Column efficiency: is a metric for peak band spreading. The bigger the number of theoretical plates, the smaller the band spread, suggesting strong column and system performance. For a good system, columns with N ranging from 5,000 to 100000 plates/meter are suitable. The formula for the calculation of N is illustrated below in the following.

$$N=16 (t R) W$$ N = Efficiency / Number of theoretical plates.

tR = Retention time of analyte, h = Height of the peak, W = Gaussiane peak width

IV. Peak symmetry factor and tailing factor: The chromatographic peak has a Gaussian form under ideal conditions. This system appropriateness criterion has mostly been proposed by regulatory organizations such as the USP and EP. The asymmetry factor is about the same in most circumstances and is only rarely accurate. Values should be in the range of 1.0-1.5. The peak symmetry is computed by utilizing the following formula,

$$As = B/A$$

Where: As = peak asymmetry factor, B = distance from the peak midpoint to the trailing edge, A = distance from the leading edge of the peak to the midpoint.

Peak purity: or peak homogeneity of the main peak to assess for the presence of impurities under the main peak.

Acceptance criteria of system suitability parameters [4]:

Sr. no.	**Parameter name**	**Acceptance criteria**
1.	Number of theoretical plates or Efficiency(N)	>2000
2.	Capacity factor(K)	<1
3.	Separation or Relative retention(α)	>1
4.	Resolution (Rs)	>1.5
5.	Tailing factor or Asymmetry(T)	<2
6.	Relative Standard Deviation (RSD)	<2

Pharmaceutical analysis has become one of the battlegrounds in the struggle due to the globalization of the drug business. As a result, the importance of medication safety issues has risen dramatically. Pharmaceutical analysis is an important subject of study to alleviate human suffering by improving medication therapy safety. Due to its great sensitivity and selectivity, HPLC combined with mass spectrometry has become the most popular approach. The spread of HPLC in the assessment of pharmaceutical formulations, which required specific procedures for demonstrating stability, was even more dramatic. In the field of pharmaceutical analysis, no other technology had spread as quickly. The HPLC procedure is used to isolate and measure active ingredients, impurities, and any degradation products. [3, 11]

Application of HPLC for estimation of some API`s: [10, 12, 13, 14, 15, 16, 17, 18, 19,]:

Drug	Column	Mobile phase	Flow rate	Detection wavelength
Albendazole	C18	Buffer of pH 3.5 : acetonitrile (70:30 v/v)	1ml/ min	224 nm
Aceclofenac	C18	Acetonitrile: Buffer (40:60 v/v, pH 6) Buffer containing 50mM orthophosphoric acid.	1ml/ min	270 nm
Atorvastatin	C18	Methanol : water (68:32 v/v, pH 3.0)	1.5ml/ min	241 nm
Amoxicillin trihydrate	C18	Phosphate buffer : methanol (50:50 v/v, pH 3.0)	1ml/ min	229 nm
Azithromycin	C18	Methanol : Buffer (90:10 v/v)	1.5ml/ min	210 nm
Albuterol sulfate	C8	Solvent A: 2.5 g of potassium dihydrogen phosphate + 2.87 g of heptane-1-sulfonic acid sodium salt per liter of water, pH 4 with orthophosphoric acid. Solvent B: acetonitrile	1ml/ min	220 nm
Buspirone	C18	0.010 M ammonium acetate (pH 4.0) : methanol (55:45 v/v)	0.3ml min	245 nm
Cetrizine	C18	A:50 mmol L^{-1} sodium dihydrogen phosphate, 5 mmol L^{-1} heptane sulfonic acid sodium salt, pH 4.2 B:acetonitrile	1.5ml/ min	214 nm
Chlorzoxazone	C18	Acetonitrile : Buffer (40:60 v/v), buffer containing 50mM orthophosporic acid (pH 6)	1ml/ min	270 nm
Colchicine	C18	Acetonitrile : water (55: 45 v/v; containing 0.5% v/v formic acid)	1ml/ min, 1.2	350nm

			ml/ min	
Dantrolene	C18	A:50 mmol L^{-1} sodium dihydrogen phosphate, 5 mmol L^{-1} heptane sulfonic acid sodium salt, pH 4.2 B:acetonitrile	1.5ml/ min	214 nm
Dicyclomine	C18	Acetonitrile : 20 mM potassium dihydrogen phosphate (70:30 v/v, pH 4)	1ml/min	220nm
Digoxin	C18	Water : Acetonitrile (72:28, v/v)	1.1ml/ min	218 nm
Duloxetine hydrochloride	C18	Acetonitrile : 0.01 M potassium dihydrogen phosphate buffer (pH 5.4, 50:50, v/v)	1ml/ min	229 nm
Entacapone	C8	Acetonitrile : 0.01% Orthophosphoric acid pH 2.5	1.0ml/ min	210nm
Ephedrine hydrochloride	C18	A: acetonitrile and water (5:95 v/v, pH 2.40) B: acetonitrile and water (40:60 v/v, pH 2.40)	1.5ml/ min	from 220 nm at start time, to 240 nm at 3.70 min and again to 220 nm at 10.70 min
Ethinyl estradiol	C18	Methanol : water (80:20 v/v)	1ml/ min	220 nm
Etoricoxib	C18	10 mM ammonium acetate buffer : Acetonitrile (65:35 v/v)	1ml/ min	235 nm
Febuxostat	C18	Acetonitrile : water (55: 45, v/v; containing 0.5% v/v formic acid)	1ml/min, 1.2 ml/ min	320nm
Furosemide	C8	Acetonitrile : 0.01M ammonium acetate	1ml/	254 nm

		buffer (pH 3.9 , 50: 50 v/v)	min	
Fexofenadine	C18	Methanol : Phosphate buffer (35:65 v/v, pH 7.4)	1ml/ min	218 nm
Gestodene	C18	Methanol : Water (80:20 v/v)	1ml/ min	220 nm
Hydroxyzine hydrochloride	C18	A: acetonitrile and water (5:95 v/v, pH 2.40) B: acetonitrile and water (40:60 v/v, pH 2.40)	1.5ml/ min	from 220 nm at start time, to 240 nm at 3.70 min and again to 220 nm at 10.70 min
Ipratropium bromide	C8	Solvent A: 2.5 g of potassium dihydrogen phosphate + 2.87 g of heptane-1-sulfonic acid sodium salt per litre of water, pH 4 with orthophosphoric acid) Solvent B: acetonitrile	1ml/ min	220 nm
Isoniazid	C18	Acetonitrile : 15 mmol L1 potassium dihydrogen phosphate buffer (pH 4.0, 11:89 v/v for the initial 4.5 min, and then it was maintained at 50:50 v/v	1ml/ min	235 nm
Isoxsuprine	C18	Acetonitrile : Methanol : 20 mM sodium dihydrogen phosphate (25:25:50 v/ v/v, pH 4.5)	1ml/ min	221 nm
Levamisole	C18	Buffer : Acetonitrile (70:30 v/v, pH 3.5)	1ml/ min	224 nm
Levothyroxine sodium	C18	0.01 M phosphate buffer : Methanol (55:45 v/v, pH 3)	1ml/ min	225 nm
Mefenamic acid	C18	Acetonitrile : 20 mM potassium dihydrogen phosphate (70:30 v/v, pH 4)	1ml/ min	220 nm
Methotrexate	C18	Water : Acetonitrile : Tetrahydrofuran	0.8m/	313 nm

		(65:30:5 v/v/v, pH 3.0)	min	
Montelukast sodium	C18	Acetonitrile :1 mM sodium acetate (pH 6.3, 90:10 v/v)	1.5ml/ min	285 nm
Moxifloxacin	C8	Methanol :18 mM phosphate buffer (pH 2.8, 62:38 v/v)	1.5ml/ min	254 nm
Papaverine hydrochloride	C18	A: acetonitrile and water (5:95 v/v, pH 2.40) B: acetonitrile and water (40:60 v/v, pH 2.40)	1.5ml/ min	from 220 nm at start time, to 240 nm at 3.70 min and again to 220 nm at 10.70 min
Paracetamol	C18	A:50 mmol L^{-1} sodium dihydrogen phosphate, 5 mmol L^{-1} heptane sulfonic acid sodium salt, pH 4.2 B: acetonitrile	1.5ml/ min	214 nm
Prednisolone	C8	Methanol : 18 mM phosphate buffer (pH 2.8, 62:38 v/v)	1.5ml/ min	254 nm
Probenecid	C18	Acetonitrile : water (55: 45, v/v; containing 0.5% v/v formic acid)	1ml/min, 1.2 ml/ min	249nm
Pyrazinamide	C18	Acetonitrile : 15 mmol L1 potassium dihydrogen phosphate buffer (pH 4.0, 11:89 v/v for the initial 4.5 min, and then it was maintained at 50:50 v/v)	1ml/ min	235 nm
Ranolazine	C18	A- buffer(0.02 M sodium dihydrogen phosphate and 2 mL of triethylamine, pH 4.5) : acetonitrile (90:10 v/v) B- acetonitrile : water (90:10 v/v)	1ml/ min	210 nm
Rifampicin	C18	Acetonitrile : 15 mmol L1 potassium	1ml/m	235nm

		dihydrogen phosphate buffer (pH 4.0 11:89 v/v for the initial 4.5 min, and then it was maintained at 50:50 v/v)	in	
Ritodrine	C18	Acetonitrile : methanol : 20 mM sodium dihydrogen phosphate (25:25:50 v/v/v, pH 4.5)	1ml/ min	221 nm
Ropinirole hydrochloride	C18	Buffer : Acetonitrile (50:50 v/v, pH 6.0)	0.5ml/ min	245nm
Rosuvastatin	C18	Methanol : water (68:32 v/v, pH 3.0)	1.5ml/ min	241 nm
Spironolactone	C8	Acetonitrile : 0.01M ammonium acetate buffer, (pH 3.9, 50: 50 v/v)	1ml/m in	254 nm
Theophylline	C18	A: acetonitrile and water (5:95 v/v, pH 2.40) B: acetonitrile and water (40:60 v/v, pH 2.40)	1.5ml/ min	from 220 nm at start time, to 240 nm at 3.70 min and again to 220 nm at 10.70 min
Tramadol	C18	Methanol : Water (13:87v/v, pH 2.5)	2 ml/ min	200 nm/

CONCLUSION:

Chromatography is analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in mixture by single analytical procedure. HPLC is a powerful analytical technique based on advanced technologies that have been used for decades. The HPLC is well established as an excellent method for in-process control and quality assurance of bulk medicines and formulations assuring people's safety, according to our examination of published data. The most common approach used was the relatively simple isocratic reversed-phase in combination with UV detection. HPLC applies to all drug classes because of the large range of stationary phases

and operation modes. HPLC is the most effective separation technique for quantitative trace analysis of toxic chemicals, impurities, high-purity product manufacturing, pharmaceutical applications, and research. With the use of HPLC, one can produce extremely pure compounds. The accuracy, precision, and specificity can all be improved by using HPLC.

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