Review on Simultaneous Estimation of Fixed Dose Combinations (FDC): System Suitability Testing (SST) Parameters of RP-HPLC Analysis

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.)

School of Pharmacy

BRAC University

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
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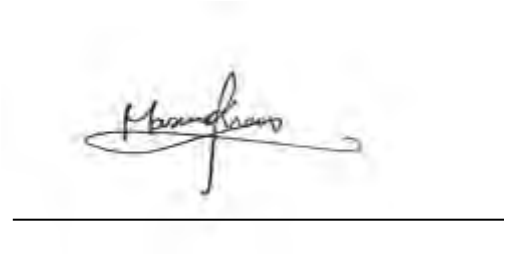

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Approval

The thesis titled "Review on Simultaneous Estimation of Fixed Dose Combinations (FDC): System Suitability Testing (SST) parameters of RP-HPLC analysis" submitted by Masuma Khan (ID-18346054), of Spring, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

This is an original work by the author that has not been previously published elsewhere. This study does not involve any kind of animal and human trial.

Abstract

This study presents the system suitability criteria for the assessment of multiple fixed dose combinations using reversed - phase chromatography. RP-HPLC is a separation technique based on the lipophilic interaction between the solute molecules in the mobile phase and the aromatic ligand. System suitability tests are utilized to confirm that a chromatographic system is suitable for independent analysis. The assay of combinational oral dosage forms using RP-HPLC was studied in this review. The objective was to determine the compliance of the assays in existing literature meets SST characteristics established by the USP recommendation. If the characteristics were not met, the cause was identified and a possible solution was proposed. Twelve articles were identified as part of this research. Reviewing the system suitability testing (SST) parameters as per USP guideline suggested, reflects any potential lack of knowledge part in of method development in developing robust methods.

Keywords: RP-HPLC, RSD, System suitability, USP, Theoretical plate number, Column.

Acknowledgment

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Table of Contents

List of Figures

List of Tables

Abbreviations

RP-HPLC: Reverse-phase high-performance liquid chromatography

HPLC: High-performance liquid chromatography

SST: System suitability testing

RSD: Relative standard deviation

USP: United States pharmacopeia

BP: British pharmacopeia

ICH: International Council on Harmonization

LOD: Limit of detection

LOQ: Limit of quantification

FDC: Fixed-dose combinations

NSAID: Non-steroidal anti-inflammatory drug

PDA: Photo-diode array

ACN: Acetonitrile

TEA: Triethyl amine

TFA: Trifluoracetic acid

SPE: Solid Phase Extraction

CDER: Centre for Drug Evaluation and Research

IPA Isopropyl alcohol

UV Ultra-viole

CHAPTER 1

1.1 INTRODUCTION

Chromatography is a vital biophysical method for separating, identifying, and purifying the components of a mixture for qualitative and quantitative investigation. In chromatography, a mobile phase helps separate molecules in a mixture that has been applied on a solid surface or inserted into a fluid stationary phase (stable phase). Molecular features such as adsorption (liquid-solid), partition (liquid-solid), affinity, or changes in molecular weight play a role in this separation process. These variations lead certain components of the mixture to spend more time in the stationary phase, moving more slowly through the chromatographic system, while other components pass quickly into the mobile phase, moving through the system more quickly, and eventually leaving the system. Liquid chromatography, Gas chromatography, Thin-layer chromatography and Ion exchange chromatography are some of the most popular chromatography techniques (Coskun, 2016).

HPLC, or high-performance liquid chromatography, is a type of column chromatography typically employed in biochemistry and analysis for the purposes of separating, identifying, and quantifying the active chemicals of interest. High-performance liquid chromatography (HPLC) is an analytical separation method involving the high-pressure flow through a column containing the stationary phase. A mixture of substances injected at one end of the column separates as it moves through the column. A stationary phase (column) is used in high-performance liquid chromatography (HPLC), and the mobile phase(s) are pumped through the column and detected at the detector. The interactions between the stationary phase, the molecules of interest, and the solvent(s) utilized determine the retention time (D. B. Patel, 2009). Compounds that have been separated are detected electronically as they elute from the opposite end of the column. In chromatography, we can use either an isocratic or a gradient elution method. For the separation of straightforward mixtures, isocratic elution, in which the solvent composition is maintained throughout the separation process, is the method of choice. Gradient elution, in which the solvent's composition shifts with time, is applicable to a wide variety of challenging solutions. Finally, there are two types of High-performance liquid chromatography: Normal-phase high-performance liquid chromatography (NP-HPLC) and Reversed-phase high-performance liquid chromatography (RP-HPLC).

1.2 TYPES OF HPLC AND OVERVIEW ON RP-HPLC

Normal phase chromatography is the approach that uses polarity to separate analytes. A polar stationary phase and a non-polar mobile phase are utilized in NP-HPLC. The polar analyte interacts with the polar stationary phase and is retained by it (Patel, 2009).

Reversed phase chromatography (RP-HPLC or RPC), the stationary phase is non-polar, and the mobile phase is polar in reverse-phase chromatography. The mobile phase is usually a mixture of water and a polar organic solvent (Patel, 2009). Hydrocarbons are an example of a non-polar bonded stationary phase (most commonly C8 and C18). A column is a small metal tube containing a phase that is used to separate the constituents of a mixture. When the column length increases, so does the retention time, resulting in a longer analysis time. A mixture of water and an organic solvent such as methanol, acetonitrile, and IPA are examples of polar mobile phases. The mobile phase must be degraded and filtered. The retention time decreases as the flow rate of the mobile phase increases, resulting in a shorter analysis time. Typically, the stationary phase is associated with a silica or silica-based system. The polar mobile phase expedites the elution of polar components, whereas the nonpolar mobile phase expedites the elution of nonpolar components. Polarity of the solvent influences the separation process. The eluent strength (the capacity of the mobile phase to elute a solute from the column) increases as the polarity of the stationary phase decreases. In the polar stationary phase, the solubility of polar solutes is increased. Due to the predisposition for pH maxima to dissolve silica gel and break the connections between silane-coating agents and the silica gel support, the pH of the mobile phase can only be regulated between approximately 2 to 8.5 pH units (G. Watson, 1999).

Biochemical separation and purification use analytical and preparative reversed phase chromatography. Proteins, peptides, and nucleic acids can be separated using reversed phase chromatography with quick recovery and resolution. The use of ion pairing modifiers in the mobile phase permits reversed phase chromatography of charged solutes like oligonucleotides and hydrophilic peptides. Preparative reversed phase chromatography is used to purify protein fragments for sequencing and recombinant protein products at large scale (Eriksson, 2018).

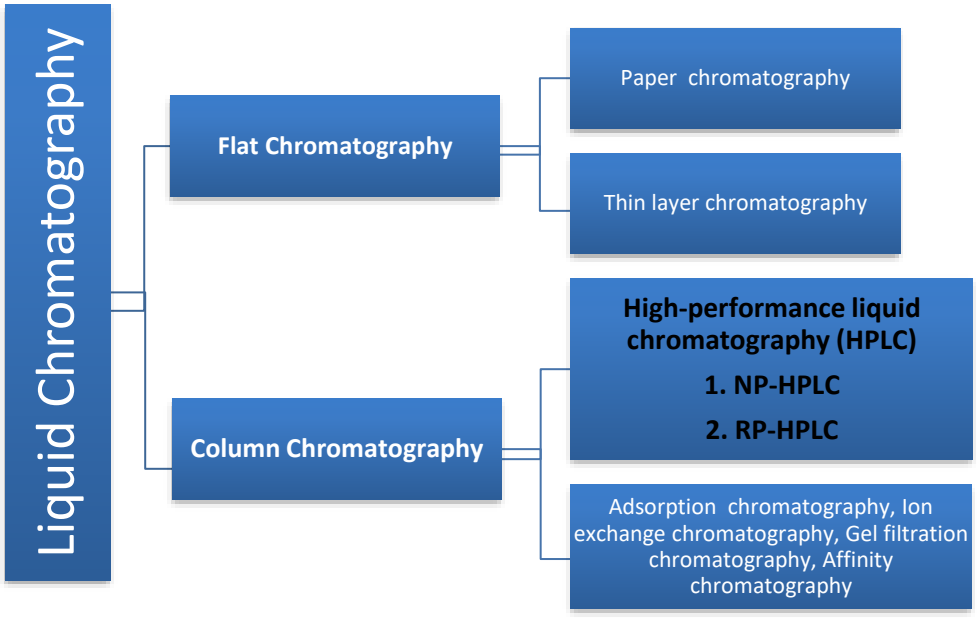

Fig-1: Types of Liquid Chromatography

1.3 EFFECTS OF PHYSICOCHEMICAL PROPERTIES ON RP-HPLC

When developing an HPLC technique, an analyte's physicochemical properties are crucial. Studying the physical characteristics of the drug molecule, for instance its solubility, polarity, pKa, and pH, is necessary for the development of chromatographic methods. Both pH and pKa are crucial elements in the construction of HPLC methods. An acidic compound is more retained with low pH mobile phase. On the other hand, a basic compound retained more in a greater pH mobile phase. In low pH mobile phase acidic drug or compound are not ionised, it is neutral and can be retained largely in stationary phase. The higher the pH more is the ionisation and there will be lesser retention. Non-ionisable analytes do not require the addition of buffers to the mobile phase. But when the analyte is ionisable, a buffer must be added to the mobile phase. For ionisable chemicals, it is necessary to know the pka of the analyte. The pKa is a property of a compound that defines how rapidly the chemical gives up a proton. The optimal pH of the mobile phase is 2 units greater than the pka of the analyte. When the pH of the mobile phase is too close to the pka of the analyte, peak splitting occurs. When bases are charged and acids are neutral, the best results can be achieved (Chrominfo: Role of PKa in HPLC Method Development, 2019).

In reverse-phase chromatography, the mobile phase is often a mixture of water and a polar organic solvent, whereas the stationary column phase is non-polar. When a mixture of components is subjected to reverse phase, we can anticipate that the hydrophilic or polar compounds will have a

very short retention time. Retention period is the length of time that the column retains the original compound. Therefore, a low retention time indicates that the substance will rapidly leave the column. And if we're dealing with hydrophobic matter, this will interact with the column to a far greater degree. The hydrophobic material in the solutions that are being separated will adhere to the column better due to stronger Van der Waals interactions and dispersion forces as they're more miscible with the stationary phase. Consequently, it will be much more difficult to push them all the way to the detector, resulting in a longer retention time. A key parameter for characterizing biological partitioning or distribution is the logarithm of the n-octanol/water partition coefficient (logP) or the distribution coefficient (logD)(Chiang & Hu, 2009). The higher the logP is, the more lipophilic the compound becomes. The logP scale can be utilized to calculate and quantify the hydrophobicity of a molecule.

$$\textbf{logP} = \boldsymbol{log}\,\frac{[\boldsymbol{solute}]\boldsymbol{octanol}}{[\boldsymbol{solute}]\boldsymbol{water}}$$

So, the logP value indicates how much more soluble an object is in a hydrophobic liquid than in a hydrophilic one (water). A partition co-efficient is a method of determining how a compound dissolves in various solvents. Because octanol is non-polar, the numerator will be very big, and logP will be positive, if the chemical is mostly soluble in it. Whereas, logP will be equal to 0 (because log1=0) if the molecule is completely soluble in both solvents. Finally, a negative value for logP indicates that the substance is more water-soluble than hydrophobic. Lipophilicity (logP) is associated to API pharmacokinetics, making it critical for drug candidate screening. According to solvophobic theory, the Rt in RPHPLC is determined by lipophilicity, or the compound's dynamic distribution between the stationary and mobile phases (Liang & Lian, 2015).

Table 1: logP scale

logP scale	Lipophilicity
logP>1	Hydrophobic
logP=0	Neutral
logP<0	Hydrophilic

When it comes to determining lipophilicity, logP is an essential parameter. The lipophilicity of a drug is what decides whether or not it will be able to pass across a lipid membrane. The log scale goes from a negative 5 all the way up to a positive 5. The logarithmic scale goes from minus 5 to plus 5. The negative numbers represent hydrophilicity, while the positive numbers represent lipophilicity. The logP can be used to predict solubility.

1.4 SYSTEM SUITABILITY TEST IN RP-HPLC

System suitability is a quantitative assessment in which properties of the instrument, technology (e.g., chromatogram synthesis), and analytical technique are examined. Collecting accurate and precise HPLC data requires a well-behaved chromatographic system. The system suitability criteria and tests are parameters that aid in the accomplishment of this objective. In addition, the system suitability test may be viewed as a test for the applicability of the mobile phase utilized throughout the study. Typically, each of them will aid in determining if the system (including the column and mobile phase) is acceptable for measuring the samples, while controls will validate quantitation accuracy. (Tiryaki et al., 2009) System suitability tests are an essential element of chromatographic procedures, and they are used to ensure that the system's resolution and repeatability are suitable for the study. Five most widely used SST parameters are Resolution, RSD, Theoretical plate number, Tailing factor and Capacity factor. (Dejaegher et al., 2021) If the results of these observations could be affected by changes in the analytical circumstances, we need to make sure to take the necessary precautions. The examination of robustness and ruggedness should lead to the establishment of a set of system suitability characteristics that can be utilized to preserve the analytical procedure's validity in all circumstances. Analytical solutions, tools, and analysts can all vary widely. The pH of the mobile phase, the mobile phase constitution, different lots or manufacturers of columns, temperature, and flow rate are examples of variables that can affect the results of a liquid chromatography experiment. The idea behind system suitability testing is that the apparatus, electronics, analytic procedures, and samples to be examined all work together and can be evaluated as a whole. The parameters of the system suitability test that must be defined for a given operation are determined by the nature of the procedure itself. They play a vital role in chromatographic techniques. All USP submissions need to adhere to the standards laid down in *Chromatography <621>*, System Suitability *(United States Pharmacopeia Convention (USP), 2013).*

The ratio of the separation between two neighbouring peak maxima and their widths is referred to as resolution. To define the separation of important pairs of constituents in complicated sample mixes, Rs should be measured. The given formula is utilized to determine resolution,

$$\textbf{Resolution, Rs} = (t_{r2}-t_{r1})/\ (1/2)\ (t_{w1}+t_{w2})$$

Here, t_{r2} and t_{r1} are retention time. t_{w1} and t_{w2} are the equivalent widths at the peaks' bases. (Tiryaki et al., 2009)

Injection precision, reported as RSD (relative standard deviation), reflects the HPL chromatograph's efficiency, which comprises the piping, column, and environmental parameters, as well as the interval the specimens are examined. It deserves to be emphasized that changes in sample preparation and production are not taken into account. (Dejaegher et al., 2021) The following equation is used to calculate %RSD,

$$\%\textbf{RSD}= KB\sqrt{n}/t_{90\%,n-1}$$

Where, K= is a constant, obtained from the following expression,

$K= (0.6/\sqrt{2}) \times (t_{90\%-5}/\sqrt{6})$,

B= Upper limit given in the definition of individual monograph-100%,

n= Number of replicate injections of the reference solution ($3 \leq n \leq 6$) and

$t_{90\%,\ n-1}$ = t at the 90% probability level with n-1 degree of freedom.

Theoretical plate number is a measure of column efficiency, or how many peaks can be identified per unit chromatogram run-time. The number of plates is a quantitative measure of column efficiency. This may be determined in a variety of ways employing several peak expanding metrics. In relation to the plate number, the plate number/meter values are frequently included. The following equation is used,

$$\textbf{Theoretical plate number, N}= 16\left(\frac{t'R}{Wb}\right)^2$$

Peak location, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and analyte molecular weight are all variables that might impact N. (Tiryaki et al., 2009)

The retention factor (k'), also known as the partition ratio or capacity factor, is the ratio of time being spent by a molecule in stationary phase to time spent in mobile phase. This is one of those factors that can be easily detected. k' is a quantity that has no unit. The following equation is used to determine retention factor, (Tiryaki et al., 2009)

$$\textbf{Retention factor, k' } = \frac{tR-t0}{t0}$$

Here, t_R= retention time of the analyte and t_0= time for mobile phase to elute/ retention time of the not retained component

Peak tailing is a well-known phenomenon that can have an impact on the accuracy estimation of a chromatographic system since peak integration based on where the peak finishes might be difficult. It is critical to know the location of the upslope and downslope, else the accuracy would suffer. The accuracy of quantitation reduces as peak tailing increases due to challenges faced by the integrator in detecting where the peal finishes and hence calculating the area under the peal. The analyst sets the integrator variables for the best estimate of the area for the peak of interest. The following equation is used to determine tailing factor,

$$\textbf{Peak tailing, T} = \frac{\mathbf{Wx}}{\mathbf{2f}}$$

Where, W_x= width of the peak found at either 5% (0.05) or 10% (0.10) from the baseline of the peak height, and f = distance between peak maximum and peak front at W_x. (Dejaegher et al., 2021)

Table 2: The USP requirements for SST parameters

System Suitability Parameter	Recommended limit
Resolution	> 2
%RSD	≤1.0% for 5 replicates
Capacity factor	> 2
Theoretical Plate Numbers	> 2000
Tailing factor	≤ 2

The USP system suitability parameter includes: capacity factor, RSD, resolution, tailing factor and theoretical plates etc. Various labs, both government and private, as well as academic institutions, perform extensive testing and evaluations on USP standards. USP partners with leading scientists and health-care professionals to create performance standards for medications, supplements, and food components. Whereas, the British Pharmacopoeia (BP) is a compilation of regulations for active pharmaceutical components and pharmaceutical products that has been released for public use. USP demands more stringent requirements for SST compared to BP and ICH. These standards enable users to conduct an objective evaluation of a material's quality. To ensure the safety and effectiveness of daily medications used by consumers, quality is essential. In addition to good practice quality guidelines and regulations and regulatory evaluation, pharmacopoeial quality standards are one of the pillars of assuring acceptable quality (Bell, 2019). On the authority of USP, the analytical performance characteristic contains 8 performance characteristics such as Accuracy, Precision, Linearity, Specificity, LOD, LOQ, System suitability and Robustness.

CHAPTER 2

METHODOGY

To begin with, 60 research articles was obtained from various sources from the following databases which includes, **Elsevier**, **Science direct**, **Research gate**, **Hindawi**, **Wiley** and **Springer link.** After reviewing those 60 papers, a total of 14 research articles were selected for further examination on the grounds that they met the criteria of being pertinent, having an appropriate dosage form, having information that was roughly up to date, and having a substantial amount of data. For this particular search, I used a unique search term, such as "Simultaneous estimation" and/or "RP-HPLC." After that, essential data from the articles were accumulated, arranged, and reconfigured in a few excel spread sheets.

CHAPTER 3

LITERATURE REVIEW

RP-HPLC assay methods of combinational drugs in tablet formulation were reviewed. Combinational drugs, sometimes known as fixed-dose combinations (FDCs), are medications that contain two or more active components but only one dosage form. Potential benefits of fixed combination preparations include greater compliance, synergy and enhanced efficacy, as well as decreased adverse effects and cost.

In table 2.1 the classes of combinational drugs and their therapeutic uses are enlisted. The physicochemical properties of the drugs are then recorded in the table 2.2. Again, in table 2.3 the chromatographic conditions for the assay are documented. Finally, in table 2.4 the system suitability parameters are listed.

Table 3: Class of combinational drugs and their therapeutic uses of drug in literature reviewed

SL No.	Drug Name	Drug Class	Uses	Reference
1	Cefixime	Cephalosporin antibiotics	Treatment of treat bronchitis, gonorrhoea and pneumonia.	(Rathinavel & Mukherjee, 2008)
	Cloxacillin	Penicillin antibiotic	Treatment of skin infections and septic arthritis.	
2	Indomethacin	NSAID	Treatment of chronic musculoskeletal pain	(Assali et al.,2020)
	Paracetamol	Analgesic and antipyretic	Relief of ever and pain reliever	
	Famotidine	H2 blocker	Treatment of duodenal ulcers	
3	Aceclofenac	NSAID	Reduction of pain in	(Vaidya et al.,

			rheumatoid arthritis	2009)
	Paracetamol	Analgesic and antipyretic	Relieves headache	
	Tizanidine	Alpha-2-adrenergic agonists	Treatment of spasms.	
4	Aspirin	NSAID	Treatment of muscle aches.	(Patel et al. 2012)
	Esomeprazole	Proton pump inhibitors	Treatment of gastroesophageal reflux disease (GERD)	
5	Clarithromycin	Macrolide antibiotic	Treatment of pharyngitis and tonsillitis	(Gangishetty et al., 2013)
	Paracetamol	Analgesic and antipyretic	Relief fever and pain	
6	Levamisole	Anthelminthic	Treatment of worm infestations in livestock.	(Sowjanya et al.,2018)
	Albendazole	Benzimidazole anthelmintic	Treatment of several worm-related illnesses	
7	Esomeprazole	Proton pump inhibitors	Treatment of gastroesophageal reflux disease (GERD)	(Urmi et al., 2022)
	Naproxen	NSAID	Treatment of tendinitis, osteoarthritis	
8	Dolutegravir	Antiviral	Treatment of HIV	(Noorbasha et al., 2020)
	Lamivudine	Reverse transcriptase inhibitor	Treatment of HIV/AIDS and hepatitis B.	

9	Rosuvastatin	HMG-CoA reductase inhibitor	Treatment of hypercholesterolemia.	(Beludari et al., 2013)
	Ezetimib	cholesterol absorption inhibitor	Treatment of hypercholesterolemia.	
10	Paracetamol	Analgesic and antipyretic	Relief fever and pain.	(Byran et al., 2010)
	Diclofenac potassium	NSAID	Treatment of menstrual cramps	
11	Cefepime Hydrochloride	cephalosporin antibiotics	Treatment of urinary tract and kidney infections.	(Tamboli et al., 2013)
	Tazobactam Sodium	Beta-lactamase inhibitor	Treatment of bacterial infections	
12	Pyridoxine Hydrochloride	Vitamin	Preventing vitamin B6 deficiency.	(Nawaz et al., 2013)
	Meclizine Hydrochloride	Antihistamine	Treatment of vertigo.	

Table 4: Physiochemical properties of the drugs

SL No.	**Drug**	**Physiochemical properties**		**Reference**
		pka	**logP**	
1	Cefixime	4.07	-0.4	(Rathinavel & Mukherjee, 2008)
	Cloxacillin	3.75	2.78	
2	Indomethacin	4.50	0.51	(Assali et al.,2020)
	Paracetamol	9.50	4.27	
	Famotidine	6.76	-0.57	

3	Aceclofenac	4.70	4.88	(Vaidya et al., 2009)
	Paracetamol	9.50	0.51	
	Tizanidine	7.49	1.72	
4	Aspirin	3.50	1.18	(Patel et al. 2012)
	Esomeprazole	4.77	0.6	
5	Clarithromycin	8.38	3.18	(Gangishetty et al., 2013)
	Paracetamol	9.50	0.51	
6	Levamisole	6.98	1.84	(Sowjanya et al.,2018)
	Albendazole	9.79	2.7	
7	Esomeprazole	4.78	2.43	(Urmi et al., 2022)
	Naproxen	4.20	3.29	
8	Dolutegravir	8.20	2.2	(Noorbasha et al., 2020)
	Lamivudine	14.29	-1.3	
9	Rosuvastatin	4.6	1.92	(Beludari et al., 2013)
	Ezetimib	9.75	4.14	
10	Paracetamol	9.50	0.51	(Byran et al., 2010)
	Diclofenac potassium	-2.1	4.26	
11	Cefepime Hydrochloride	2.82	-4.2	(Tamboli et al., 2013)
	Tazobactam Sodium	2.86	-1.4	
12	Pyridoxine Hydrochloride	9.107	-0.95	(Nawaz et al., 2013)
	Meclizine Hydrochloride	9.108	6.39	

Table 5: Chromatographic conditions for the assay

Drug	Name of the HPLC system	Stationary Phase	Mobile Phase	Detector	Reference
Cefixime Cloxacillin	SHIMADZU prominence	C18 column (5µm, 25 cm x 4.6 mm, i.d)	Phosphate buffer (pH 5.0), acetonitrile and methanol1,2 in the ratio (80:17: 3 v/v), isocratic	SPD 20A UV-visible absorbance detector	(Rathinavel & Mukherjee, 2008)
Indomethacin Paracetamol Famotidine	Waters 1525, Singapore, Binary HPLC pump	C18 column 5 µm, 4.6 × 250 mm analytical column	acetonitrile: sodium acetate buffer 60 : 40 at a flow rate of 1.4 mL/min and pH 5	Waters 2298 photodiode Array Detector	(Assali et al.,2020)
Aceclofenac Paracetamol Tizanidine	Agilent 1100 series HPLC	A hypersil C18 column (250 mm x 4.6 mm, 5 µm particle)	A mixture of phosphate buffer pH 7.0: acetonitrile in the ratio (40:60) v/v, isocratic	Photo-diode array detector	(Vaidya et al., 2009)
Aspirin Esomeprazole	An isocratic HPLC system (Analytical Technologies Limited)	HyperChrom ODS-BP C18 column (200 mm × 4.6 mm; 5.0 $\mu\mu$m)	Acetonitrile : methanol : 0.05 M phosphate buffer at pH 3 adjusted with	UV 2230 plus detector system	(Patel et al. 2012)
Clarithromycin Paracetamol	Shimadzu-LC 20AT	C18 column (250 mm × 4.6 mm i.d., particle size 5 μm)	Monobasic phosphate buffer (0.05 M) along with 1-octane sulphonic acid sodium salt monohydrate	UV detector	(Gangishetty et al., 2013)
Levamisole Albendazole	Shimadzu HPLC, Class VP series	INERTSIL column, C18 (150x4.6 ID),5 micrometer	Mixed Phosphate bufer (KH2PO4+K2HPO4): Acetonitrile 30:70 (Gradient}	UV-detection at 224 nm	(Sowjanya et al.,2018)
Esomeprazole Naproxen	Prominence LC-20AD HPLC (Shimadzu, Japan)	C18-ace-EPS, 250×4.6 mm ID, and 5 µm particle size column	Phosphate bufer of pH 6.8 and MeOH at a ratio of 50: 50 (% v/v)	Photodiode array (PDA) detector SPD-M20A	(Urmi et al., 2022)

		(Bischof, Germany)			
Dolutegravir Lamivudine	HPLC-Waters alliance (Model-2695)	Inertsil ODS 250 × 4.6 mm, 5 µm	Phosphate buffer solution pH 3.0, acetonitrile and methanol were taken within the ratio of 50:20:30% v/v/v (Isocratic)	2996 PDA detector	(Noorbasha et al., 2020)
Rosuvastatin Ezetimib	LC Waters (Waters, Milford, MA, USA	Water's C18 column (250 mm x 4.6 mm, 5 µm particle)	Acetonitrile: water: 0.02 M phosphate buffer pH 8 (40:10:50 v/v) quaternary gradient system	Photo diode array (PDA) detector	(Beludari et al., 2013)
Paracetamol Diclofenac potassium	Shimadzu liquid chromatographic system	Phenomenex LUNA C18 (25 cm x 4.6 mm i.d., 5µ)	acetonitrile & sodium dihydrogen ortho phosphate (70:30 v/v)	SPD M-10AVP photo diode array detector	(Byran et al., 2010)
Cefepime Hydrochloride Tazobactam Sodium	Agilent G1315D diode array detector.	PrincetonSPHER-100 C-18 (250 mm × 4.6 mm i.d., 5 μm) 5 μm particle size.	25 mM potassium dihydrogen phosphate buffer , pH 6.2 and acetonitrile (94 : 6, v/v)	Agilent G1315D diode array detector.	
Pyridoxine Hydrochloride Meclizine Hydrochloride	Shimzadu prominence LC-20AD HPLC,	Hichrom C18 (250 × 4.6mm ID, 5 μm particle size)	(Isocratic) A mixture of buffer, acetonitrile, and	Photodiode array (PDA) detector	(Tamboli et al., 2013)

Table 6: System suitability testing parameters of the assay methods

SL No.	Drug	System Suitability				
		%RSD	N	Rs	k'	T
1	Cefixime	0.69	8826	-	-	1.27
	Cloxacillin	0.77	10366	6.9	-	1.24
2	Indomethacin	<2	2160	6.7	5.4	1.10
	Paracetamol	<2	2101	6.8	2.1	0.90
	Famotidine	<2	1418	1.2	1.67	1.10
	Codrug	<2	6499	7.5	10.42	1.0
3	Aceclofenac	0.52	3291	-	-	1.36
	Paracetamol	0.09	7841	5.5	-	1.64
	Tizanidine	0.77	1129	4.2	-	2.48
4	Aspirin	0.14–0.38 (n=3)	3063	-	5.09	1.36
	Esomeprazole	0.38–0.83 (n=3)	2535	4.6	3.29	1.41
5	Clarithromycin	1.40	29044	-	-	0.83
	Paracetamol	0.36	48040	7.99	-	1.24
6	Levamisole	0.22	-	high	-	1.57
	Albendazole	0.46	-	-	-	1.57
7	Esomeprazole	0.001	6400	-	1.50	1.06
	Naproxen	0.21	6251	8.25	3.23	1.00
8	Dolutegravir	0.80	7643	22.51	-	1.34

	Lamivudine	0.70	55924	-	-	1.12
9	Rosuvastatin	1.07	-	-	-	-
	Ezetimib	1.11	-	-	-	-
10	Paracetamol	within the range	2085	2.23	-	-
	Diclofenac potassium	within the range	3015	2.56	-	-
11	Cefepime Hydrochloride	0.48	10505	-	2.54	1.16
	Tazobactam Sodium	1.15	12957	-	3.63	1.00
12	Pyridoxine Hydrochloride	1.06 (n=6)	5198	-	-	1.29
	Meclizine Hydrochloride	1.01 (n=6)	8127	-	-	1.53

Note: %RSD= Relative standard deviation, N= Theoretical plate, Rs= Resolution, CF= Capacity factor and T= Tailing factor

During my analysis of the research articles and their SST values, I discovered that only three of the articles by Assali , D. Patel and Urmi included all five parameters. They all do not satisfy the standards recommended as well as the limitation (following Table 2).

RSD (Relative Standard Deviation) value was mentioned in 10 out of 12 of the article. That makes 83% of showed RSD. Collectively the articles satisfied the agreeable range of RSD. But the assay developed by Assali and Byran did not mention the exact value of RSD, rather they pointed it to be within the range which was ambiguous.

Table 7: RSD requirements

%RSD	n (replicate injections)
≤ 2%	5
>2%	6

RSD or in other word Area variations has multiples reasons to elevate unreasonably. These could be due to variations in Column Temperatures (especially evident in ion exchange systems). Or maybe, injecting too much mixture material in one column. Generally, retention periods will decrease when

the amount of solute deposited onto the column is greater than the column's storage capacity. The solvent used in the sample cannot be used in the mobile phase. The injector efficiently administers the specimen with very little solvent disruption. Variable, fixed, and syringe-type injectors are used in HPLC systems. Leaks, blocked capillary tubes, and worn seals are straightforward to notice and fix. Pre-column filters prevent column frit blockage caused by injector seal degradation. Other difficulties, such irreproducible injections, are trickier. Inadequately loaded sample loops, unsuitable injection fluids, or limited sample solubility can produce variable peak heights, split peaks, and broad peaks. Mobile phase should be used whenever possible. Ensure the injection solvent is weaker than the mobile phase. (Small molecule HPLC, HPLC Troubleshooting Guide, n.d.)

Capacity factor or Retention factor was presented in four of the articles from 12 studies, Assali , D. Patel , Urmi and Tamboli and two of them meet the SST parameter except for Urmi and Assali. So, approximately 33% publications mentioned capacity factor, which is deficient and unsuitable. From reviewing these four articles, the method developed by Assali & Urmi displays a capacity factor of 1.67 for Famotidine and 1.50 for Esomeprazole respectively, which is lower than the recommended limit as per USP (k=>2). A high k' value indicates that the sample is highly retained and has spent a significant amount of time interacting with the stationary phase. A high retention factor is usually desirable. Famotidine is a weakly basic drug having a pka of 6.76 and logP of -0.57 which makes it hydrophilic. For the mobile phase a mixture of acetonitrile: sodium acetate buffer 60: 40 at a flow rate of 1.4 mL/min and pH 5 was used. The pH of sodium acetate buffer is 5 so it is an acidic buffer. Because of this acidity it cannot retain famotidine longer as famotidine is a weakly basic hydrophilic drug it gets ionised fairly easily in the mobile phase. The acidic buffer repels basic drug and it gets elutes very early. Because of that the k value is 1.67. The k value is independent on flow rate and column length so we can assume there is no issue with flow rate and column length. Esomeprazole is a weak acid with pka of 4.77. The mobile phase used for the Esomeprazole was phosphate buffer pH 7.0: acetonitrile (40:60 v/v). The pH of a phosphate buffer is 7 at 25°C which mean it is a weak acid. As pka fewer than 8 is said to be an acid and pka above 8 is considered as a base. As stationary phase and mobile phase both are acidic, the stationary phase should not be ionised in the mobile phase. One possible explanation for the low retention factor is the type of buffer that was used. In D. Patel's analysis of fixed dose combinations, Aspirin, and Esomeprazole, the mobile phase was modified using Acetonitrile (ACN). There are negative impacts of ACN on phosphate buffers. Phosphate buffers exhibit highly limited solubility in acetonitrile. Moreover, ACN is not chemically inert; it is

slowly hydrolysed and interacts with strong acids (Rudakov et al., 2018). As I have mentioned before, esomeprazole is an acidic drug so it might react with that. Another reason according to G. Watson, the biggest impacts of pH change in the mobile phase are detected within 2 pH unit on each side of the pKa value of the drug, i.e., where the partition coefficient of the partially ionized drug ranges between 99% and 1% of the partition coefficient of the unionized drug. Here the mobile phase is 2.02 units greater than the stationary phase pka, which means it is almost completely ionised in the mobile phase. There might be some other reasons as well for lowered capacity factor for instance, the mobile phase might not be filtered before sonication, general elution problem, unbalanced columns, carryover, inadequate column volumes, incorrect operating or storing conditions, dirty samples and pump slipping and etc. To increase the capacity factor of famotidine we can use a weakly basic buffer such as, ammonium chloride buffer with a pH of 9.0 as the mobile phase. To prevent low capacity factor for Esomeprazole, we can use a weakly acidic buffer such as, an acetic acid buffer with an organic solvent. We can adopt for other organic modifier with phosphase buffer e.g. methanol, ethanol, water etc. Here are some other ways to increase capacity or retention factor: Using a weaker solvent (changing polarity). When the solvent strength of the mobile phase is low, solutes spend a proportionally larger amount of time in the stationary phase. This makes their capacity factors swing up. By changing the pH, the polarity of the analyte can be changed. Using a stronger stationary phase (changing the polarity) Changing the temperature of the column. At lower temperatures, the vapour pressure of a solute is lower, so it stays in the stationary phase longer and takes longer to elute. Proteins and phospholipids can stick to the head of the HPLC column and form clumps. To fix this, use a guard column and change it when the peaks move. Pump slipping can be fixed by cleaning and replacing the seals on the heads of the LC pumps.

Resolution was indicated in seven of the articles by, Assali, Vaidya, D. Patel,B. Patel, S. Gangishetty, Noorbasha & Byran . Specifically we can also say that almost 58% studies acknowledged resolution parameter. From these seven articles six of them satisfy the standards recommended as well as the limitation (following Table 2) except for the assay developed by Assali. Resolution shown for the drug Famotidine is 1.2 which is lower than the recommendation (>2). In the assay developed by Assali, peak separation between of Paracetamol and Famotidine is 1.2. For this lesser resolution, there could be a few reasons such as impurities in the sample injector or column, the age of the column, not cleaning the column with acetone properly and air bubbles in the stationary phase etc. We can use longer columns and other methods to get a higher resolution. C18 is an example. Peak tailing can be

cut down. We can improve resolution by raising the temperature. Scaling back on the size of the particles in the stationary phase also helps. By reducing particle size (e.g. to 1.9), efficiency, resolution, and surface area are all improved. The concentration of the buffer should be between 10mM and 50mM. When the concentration is high, the buffer will become thicker. Because of this, the back pressure will also go up. Since Famotidine's pka is 9.74, the pH of the mobile phase should be at least 7-8. If the flow rate is slowed down, the resolution should be better. Better resolution can be achieved by lowering the amount of sample injected or the concentration of the sample.

Theoretical plate numbers was represented in ten articles apart from by the method developed by Sowjanya and Beludari. 83% of the article has mentioned theoretical plate number (N). Upon reviewing the articles, they all meet the standard requirement by USP except for these two studies by, Assali and Vaidya . As per Assali , the theoretical plate number for Famotidine is 1418 and for Vaidya theoretical plate number for Tizanidine is 1129, which is lower than 2000 by USP standards. The following variables have a strong to moderate impact on the number of theoretical plates: dimensions of the particle (very strong impact), dead volume of interconnecting tube (very strong impact), gradient's profile (strong impact), column's temperature (strong impact), size of column (strong impact), the stationary phase's makeup (moderate impact), make-up of the mobile phase (moderate impact), the particle's shape (moderate impact), shape uniformity of the particle (moderate impact) and so on. In HPLC, a theoretical plate is a fictional domain or stage where two phases, the stationary phase and the liquid mobile phase, reach a state of balance. When there are more theoretical plates in a column, there are more ways for the stationary and mobile phases to balance out. This makes for a better separation (Miller, 2020). According to (Deepak, 2015), in column chromatography, the theoretical plates are directly related to how well the column works. So, it can help improve the resolution, which is directly related to the square root of the number of theoretical plates when all other factors remain the same. This means that when the number of plates goes up by four, the resolution will go up by a factor of two.

Tailing Factor was found in ten journals which makes it approximately 83% affirmatory. Beludari and Byran did not includ tailing factor in their assays. All the mentioned T_f was satisfactory in accordance with USP. According to a new research article (What Causes Peak Tailing in HPLC, n.d.), While producing an HPLC method, it's crucial that the peaks have a "decent" Gaussian shape so that they can be measured and the method can be used over and over again. For the optimal outcomes, choose an HPLC column with a bonded phase that is capped at one end. If ones compounds are basic,

users might want to add TEA (Triethyl amine) to your mobile phase. If the compounds users are working with are acidic, they might want to add TFA (trifluoracetic acid) to thier mobile phase. Both modifiers will stop tailing because they will compete with the sample for ion exchange sites on the surface (free silanols). There are a few ways to keep peak tailing from happening: work at a lower pH level, by using a column with a lot of inactivity, contemplate the risk of too mass overload, scrutinize on the chance of column bed deformation, when looking at basic compounds, one should work at a high pH and utilize a sample clean-up steps etc. Solid Phase Extraction (SPE) can be used to get rid of any contaminants that are getting in the way. Employing one or all of these methods can fix the problems that peak tailing causes in chromatograms.

CHAPTER 4

CONCLUSION

The purpose of this literature review was to validate the robustness of the methodology for the assay of combinational oral dosage forms. Reviewing the system suitability testing (SST) parameters as per USP guideline suggested reflects any analytical or instrumental defects in the assay. System suitability tests are utilized to confirm that a chromatographic system is suitable for independent analysis. Nonetheless, the objective was not merely to identify the shortcomings, but also, if possible, to promote any positive alternative. Even though selected twelve publications were compiled from scientific and well recognized sources including Hindawi, Springerlink, Science Direct, and Wiley, the data was not astounding. The literature review would have benefited from more publications, which would have created more data, allowing for a better understanding of the fundamentals of RP-HPLC, including the instruments used, the optimal column size, buffer preparation, and SST validation. However, a large number of publications were discarded due to access restrictions in many publications, inability to gain access of the paid papers, failure to provide any dosage forms other than oral, and inability to obtain authorization for some restricted publications.

This literature review of simultaneous estimation of combinational drugs using RP-HPLC and study of their system suitability parameters is crucial for designing a new assay methodology, evaluating the method's robustness, and determining whether the analytical method is suitable for the intended analysis.

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APPENDIX

A Validated RP – HPLC Method for Simultaneous Estimation of Cefixime and Cloxacillin in Tablets.

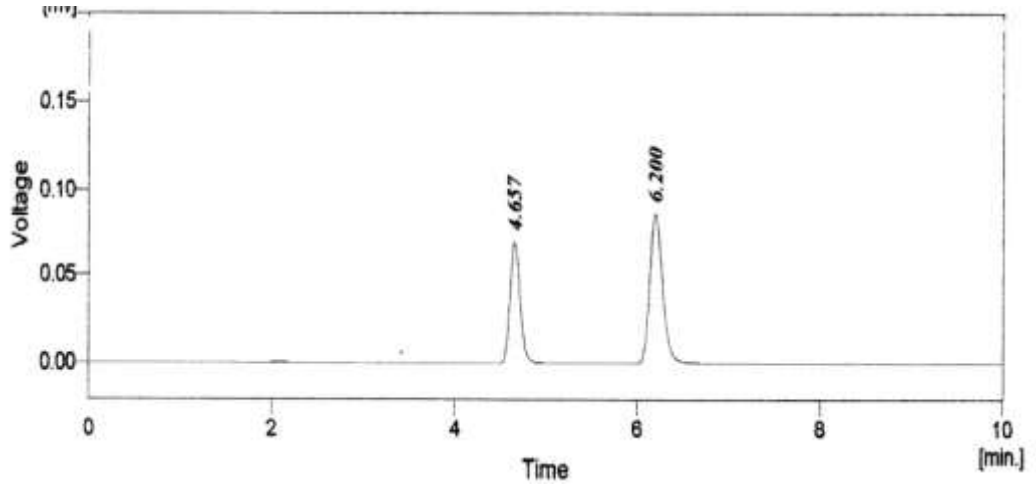

Fig: Chromatogram of Cefixime and Cloxacillin (Rathinavel & Mukherjee, 2008)

1. RP-HPLC Method Development and Validation of Synthesized Codrug in Combination with Indomethacin, Paracetamol, and Famotidine.

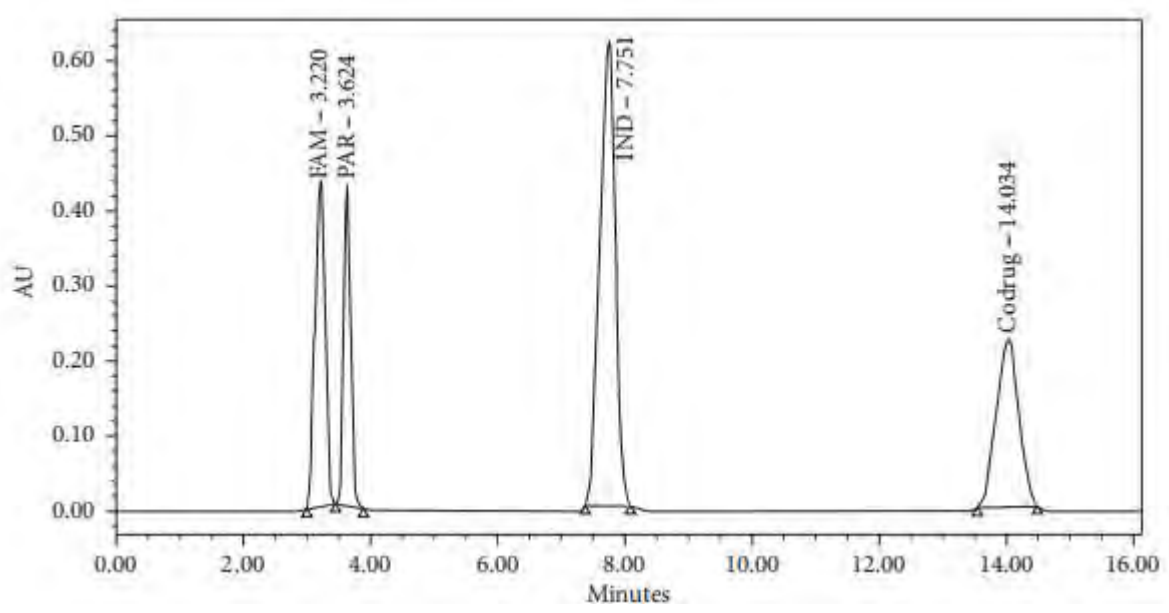

Fig: Chromatogram of Indomethacin, Paracetamol, and Famotidine. (Assali et al.,2020)

2. Simultaneous RP HPLC Determination of Aceclofenac, Paracetamol and Tizanidine in Pharmaceutical Preparations.

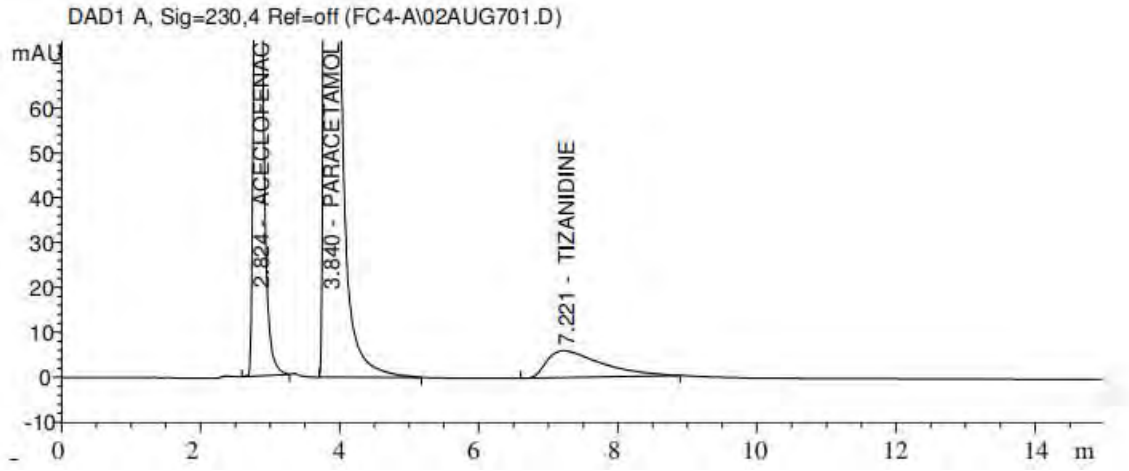

Fig: Chromatogram of Aceclofenac, Paracetamol and Tizanidine. (Vaidya et al., 2009)

3. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Aspirin and Esomeprazole Magnesium in Tablet Dosage Form.

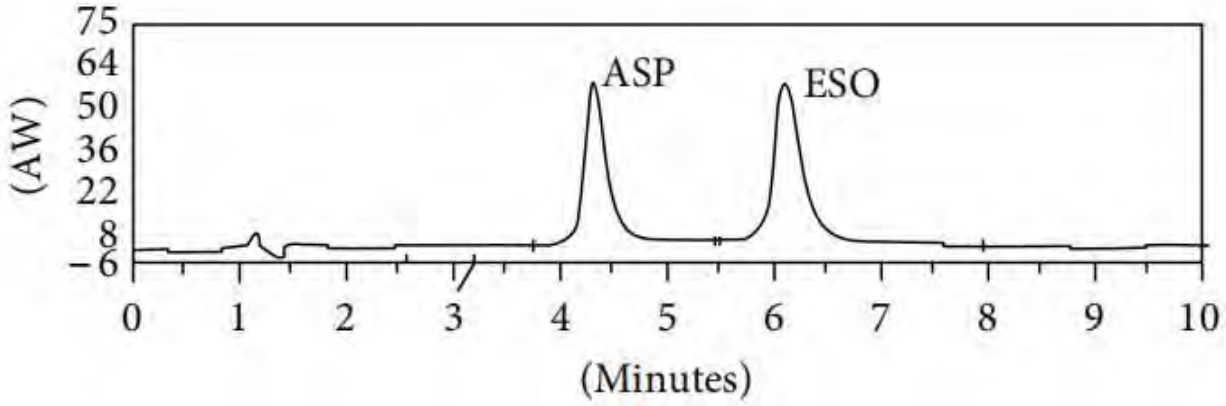

Fig: Chromatogram of Aspirin and Esomeprazole. (Patel et al. 2012)

4. RP-HPLC Method Development and Validation for Simultaneous Estimation of Clarithromycin and Paracetamol.

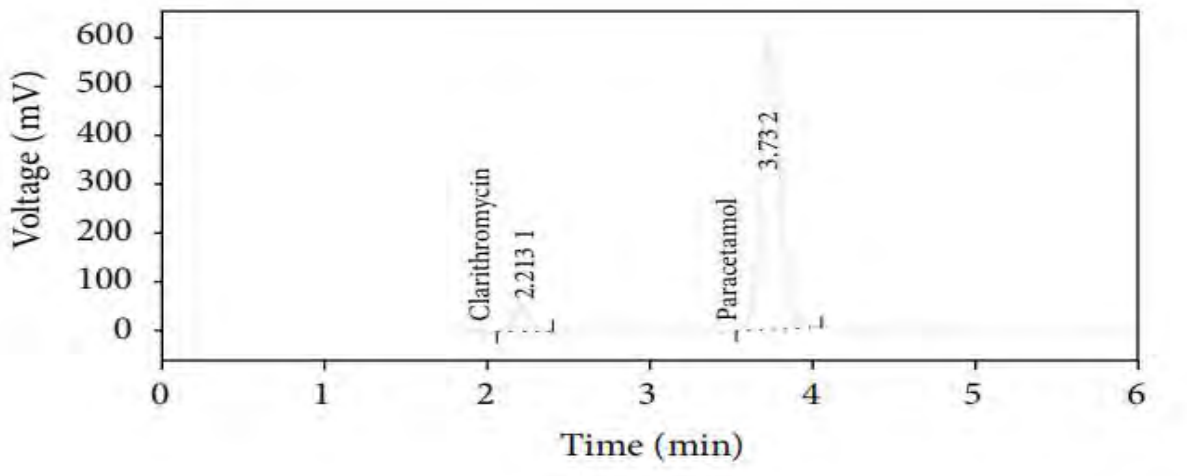

Fig: Chromatogram of Clarithromycin and Paracetamol. (Gangishetty et al., 2013)

5. Development of RP-HPLC Method for the Simultaneous Quantitation of Levamisole and Albendazole: Application to Assay Validation.

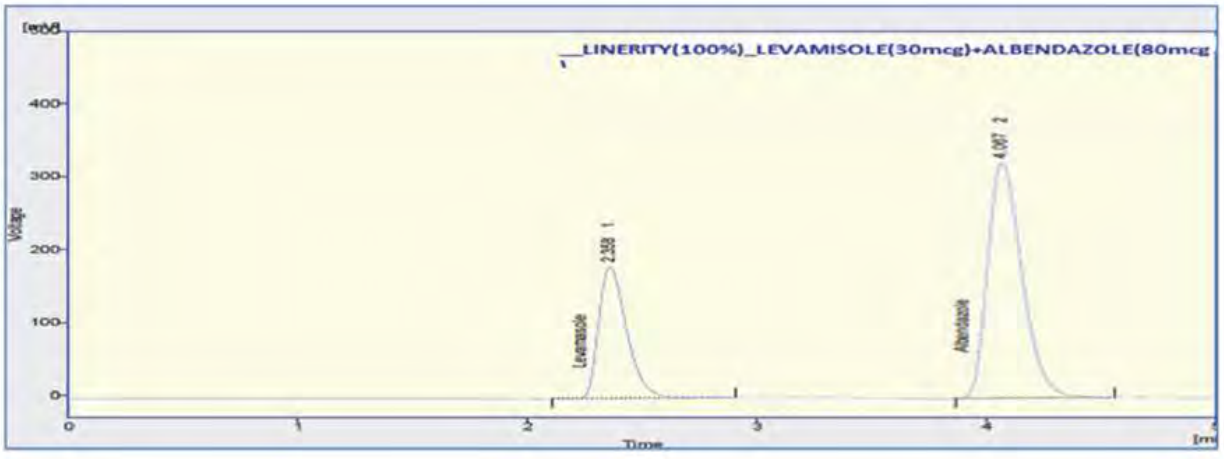

Fig: Chromatogram of Levamisole and Albendazole. (Sowjanya et al.,2018)

6. Analytical quality by design approach to RP-HPLC method development and validation for simultaneous estimation of esomeprazole and naproxen in modifed-release dosage form.

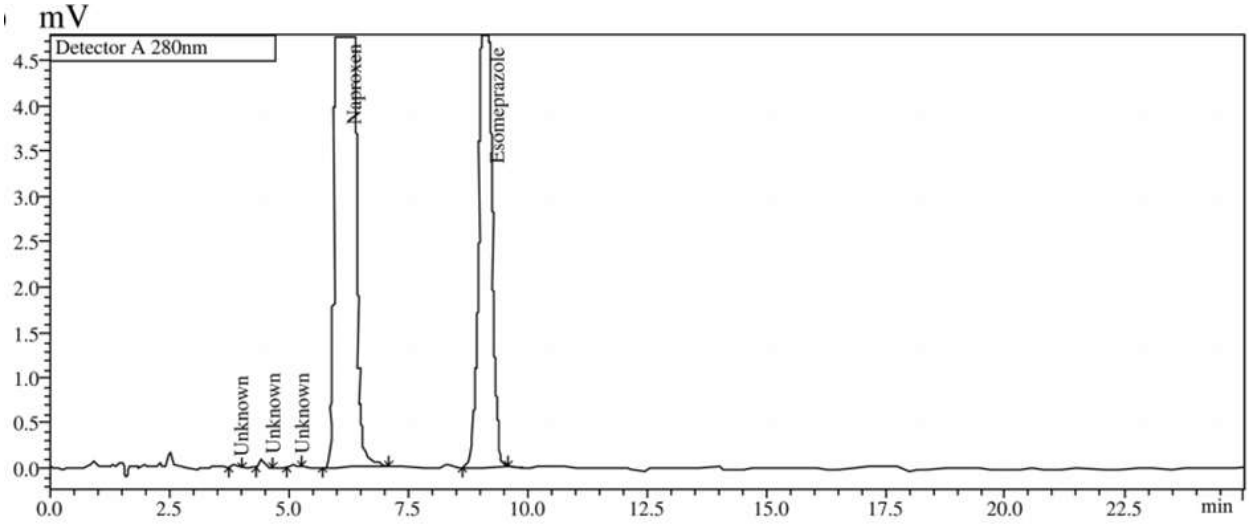

Fig: Chromatogram of esomeprazole and naproxen. (Urmi et al., 2022)

7. A new validated stability-indicating RPHPLC method for simultaneous quantification of dolutegravir and lamivudine in bulk and pharmaceutical dosage form.

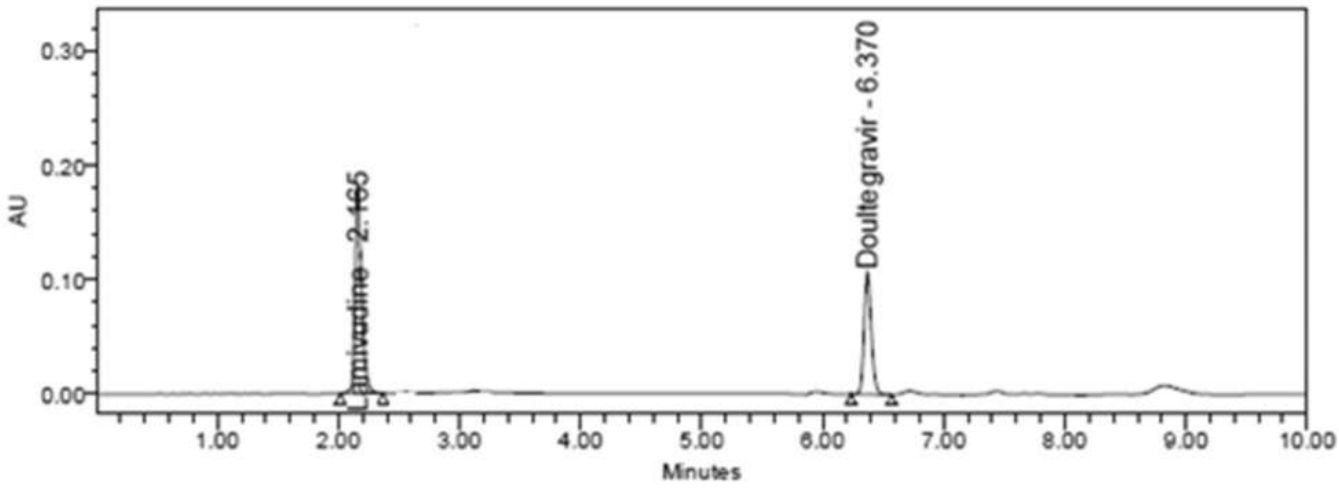

Fig: Chromatogram of dolutegravir and lamivudine. (Noorbasha et al., 2020)

8. RP-HPLC method for simultaneous estimation of Rosuvastatin and Ezetimibe from their combination tablet dosage form.

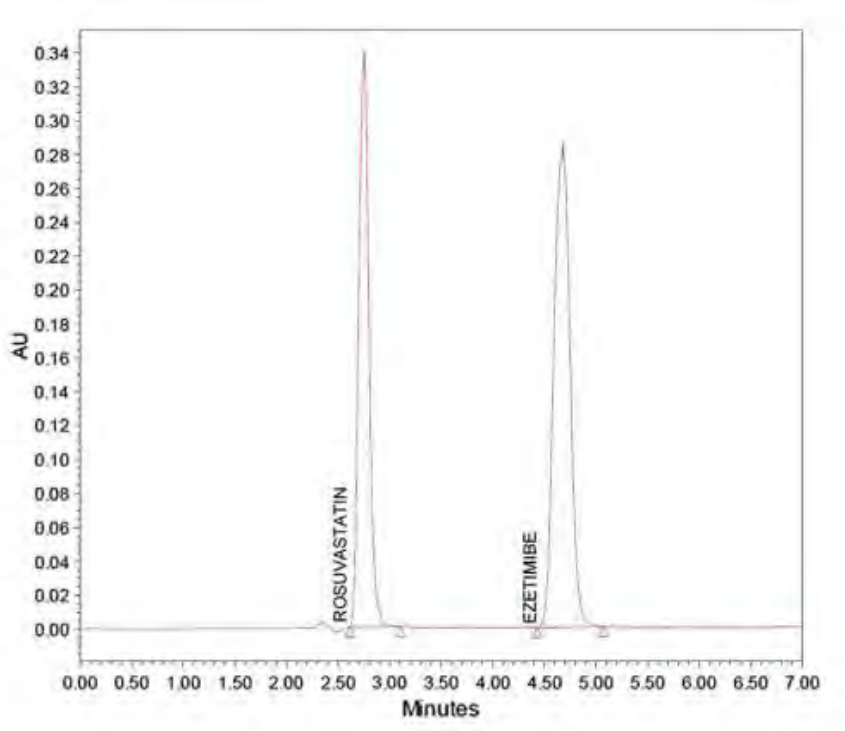

Fig: Chromatogram of Rosuvastatin and Ezetimibe. (Beludari et al., 2013)

9. A validated RP-HPLC method for simultaneous estimation of Paracetamol and Diclofenac Potassium in Pharmaceutical formulation

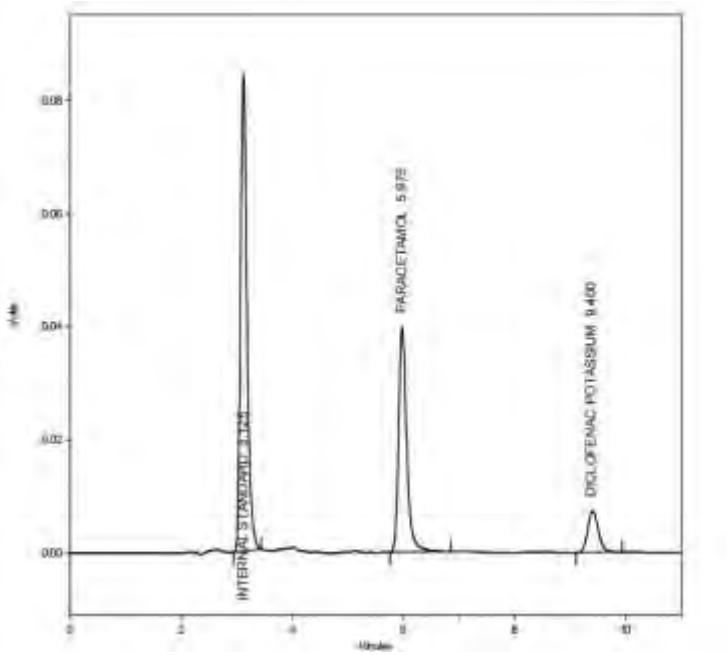

Fig: Chromatogram of Paracetamol and Diclofenac Potassium. (Byran et al., 2010)

10. RP-HPLC Method for Simultaneous Estimation of Cefepime Hydrochloride and Tazobactam Sodium in Bulk and Pharmaceuticals.

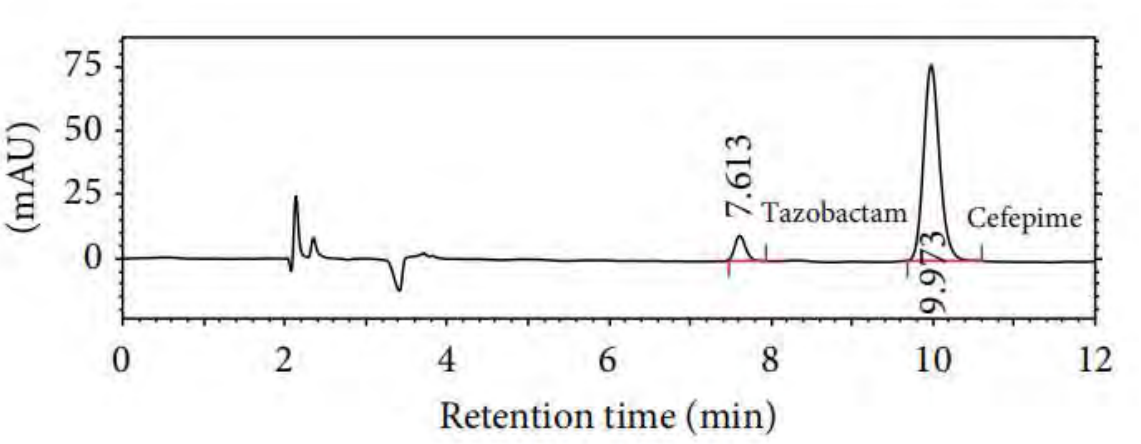

Fig: Chromatogram of Cefepime Hydrochloride and Tazobactam Sodium. (Tamboli et al., 2013)

11. A New Validated Stability Indicating RP-HPLC Method for Simultaneous Estimation of Pyridoxine Hydrochloride and Meclizine Hydrochloride in Pharmaceutical Solid Dosage Forms.

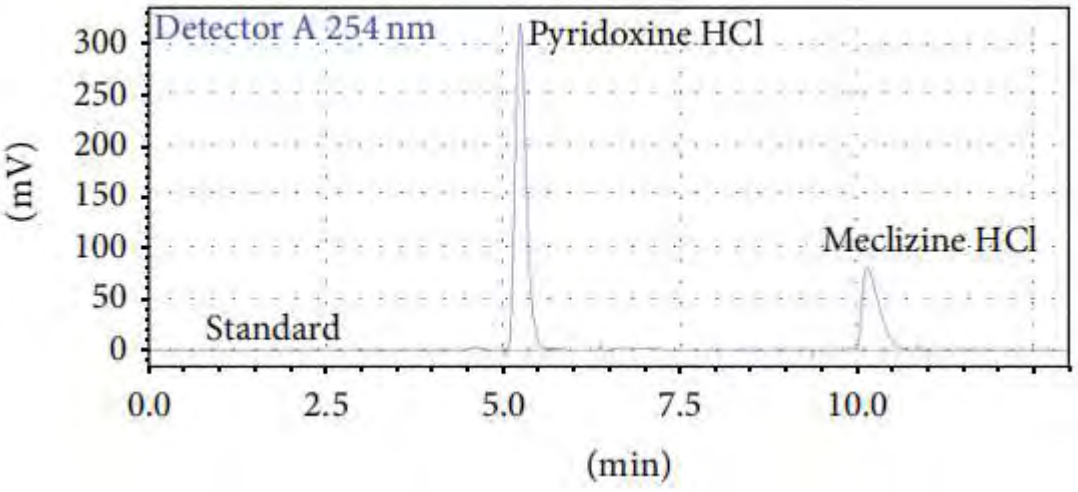

Fig: Chromatogram of Pyridoxine Hydrochloride and Meclizine Hydrochloride. (Nawaz et al., 2013)