DEVELOPMENT OF HPLC AND UFLC METHODS USING FUSED CORE TECHNOLOGY COLUMNS FOR ANALYSES OF SOME DRUGS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis, entitled **"Development of HPLC and UFLC Methods Using Fused Core Technology Columns for Analyses of some Drugs"** in fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Chemistry and submitted in School of Basic and Applied Sciences, Galgotias University, Greater Noida is an authentic record of my own work carried out during a period from January, 2013 to DECEMBER, 2017 under the supervision of Dr. ARVIND KUMAR JAIN and Dr. IMRAN ALI.

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other University/Institute.

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ABSTRACT

The research work carried out involves separation and identification of cardio vascular drugs, anti-diabetic drug and anti-histamine drugs in human plasma using solid phase extraction (SPE) and high performance liquid chromatography (HPLC). The cardiovascular drugs studied are amiloride HCl, metoprolol succinate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan. The studied anti-diabetic drugs are metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide. The studied anti-histamine drugs are phenylephrine HCl, cetirizine HCl, loratidine HCl, montelukast sodium and ebastine.

The calculated percentage recoveries of the cardiovascular drugs from plasma indicated that the values of the percentage recoveries of amiloride HCl, metoprolol succinate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan were 60, 65, 30, 10, 30, 10, 10, 10, and 100%, respectively. The remaining amounts of these drugs (bound to plasma proteins) were 40, 35, 70, 90, 70, 90, 90, 90, and 90%, respectively. The values of retention, separation and resolution factors were ranged from 0.19-3.40, 1.20-3.60 and 2.43-12.37, respectively. The reported SPE and HPLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of amiloride HCl, metoprolol succinate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan in human plasma.

The percentage recoveries of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were determined by doing the blank experiments. The intended percentage recoveries of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide in laboratory synthesized samples in water were 80, 82, 77, 87, 83, 85, 86, and 88%, correspondingly. These values in plasma were 22, 20, 21, 19, 16, 12, 10, and 17%, correspondingly. Low values in the plasma samples were due to the binding of these drugs with plasma proteins. The values of the retention, separation and resolution factors were ranged from 0.07 to 9.14, 1.44 to

4.21 and 2.15 to 18.66, correspondingly. The identification of the separated drugs was determined by running and comparing the retention times of the individual metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide molecules, correspondingly. It was observed that there was no additional peak in the plasma samples, which established the selectivity of the SPE method. From the results it was concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide in human plasma.

The percentage recoveries of phenylephrine HCl, cetirizine HCl, loratidine, montelukast sodium and ebastine were determined by doing the blank experiments. The intended percentage recoveries of phenylephrine HCl, cetirizine HCl, loratidine, montelukast sodium and ebastine in laboratory synthesized samples in water were 80, 78, 85, 94 and 71%, correspondingly. These values in plasma were 10, 12, 15, 06 and 29%, correspondingly. Low values in the plasma samples were due to the binding of these drugs with plasma proteins. The values of the retention, separation and resolution factors were ranged from 2.00 to 11.00, 1.15 to 2.31 and 1.00 to 6.07, correspondingly. The identification of the separated drugs was determined by running and comparing the retention times of the individual phenylephrine HCl, cetirizine HCl, loratidine, montelukast sodium and ebastine molecules, correspondingly. It was observed that there was no additional peak in the plasma samples, which established the selectivity of the SPE method. From the results it wa concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of phenylephrine HCl, cetirizine HCl, loratidine, montelukast sodium and ebastine in human plasma.

The reported SPE, HPLC and UFLC methods for the separation and identification (analyses) of cardiovascular, anti-diabetic and anti-histamine drugs were selective, efficient, rugged, economic, eco-friendly and reproducible in human plasma. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported drugs. Besides, the absence of any new peak established no metabolic product of these drugs in human plasma. The separation

and identification of these drugs are reported first time so far. The developed SPE, HPLC and UFLC methods were applied successfully for monitoring these drugs into human plasma. Therefore, SPE, HPLC and UFLC methods can be applied for the analyses of these drugs in any plasma sample.

Dedicated to My Mother

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(***Kamlesh Kumar Dutta***)

CONTENTS

List of Figures

List of Tables

List of Publications

1. Imran Ali, **Kamlesh K. Dutta**, A.K. Jain, Zeid A. Alothman and Abdulrahman Alwarthan, Synchronized Fast SPE and UFLC methods for the analyses of eight antidiabetic drugs in human plasma, *Comb. Chem. & High Through. Screen.*, 20: 208-214 (2017).

2. Imran Ali, **Kamlesh K. Dutta**, A.K. Jain, Mohd. Asim and Syed Dilshad Alam, Simultaneous and Fast SPE-HPLC analyses of nine anti-hypertensive drugs in human, plasma, *Am. J. Adv. Drug Deliv.*, 3: 123-134 (2015).

3. Imran Ali, N. Nagae, V.D., Gaitonde, A.K. Jain and **K.K Dutta**, Ultra fast HPLC on superficially porous columns for pharmaceutical analyses, International Symposium on Current Trends and Future Prospects in Pharmaceutical Sciences, Pokhra University, Nepal, July, 6-7 (2014).

4. **K.K Dutta**, Imran Ali, and A.K. Jain, SPE and UFLC of anti-histamine drugs in human plasma, *Curr. Drug. Ther*, Communicated (2017).

1.1. CHROMATOGRAPHY:

Chromatography is a valuable technique for the separation, purification and identification of the constituents of a mixture. This technique was first invented by Mikhail Tswett, a botanist in 1906 in Warsaw for the separation of colored substance into individual components [1]. Since then, this technique has undergone a lot of modifications and, nowadays, various types of chromatographic modalities are in use to separate almost any given mixture of compounds. The term chromatography derived from the Greek words Khromatos (color) and Graphos (writing) means to write. The general term chromatography is used to describe a wide range of techniques, which are used for the separation, purification and identification of substances, which are present only at very low concentration in a mixture. Mainly chromatography works involves two phases i.e. stationary and mobile phases. The former one is the solid support, which retains components at different extent and the later is mobile phase, which help in migration of the compounds. In TLC, the stationary phase is in the form of thin layers while it is in the form of packed column in HPLC. The mobile phases in both modalities are pure solvent or a mixture of solvents. They may be normal and reversed phase modes depending on the nature of stationary and mobile phases. In normal phase chromatography, the stationary phase is more polar than mobile phase and vice versa in case of reversed phase chromatography.

There have been many definitions of chromatography. The one that seems most satisfactory may be defined as “Chromatography is separation technique applicable to essentially molecular mixtures which relies on distribution of the mixture between

stationary and mobile phases" [2]. Another definition of chromatography which was given by Williams and Weil is "Chromatography is meant those processes which allow the resolution of mixtures by effecting separation of some or all of their components in concentration-zones on or in phases different from those in which they are originally present, irrespective of nature of force or forces causing the substances to move from one phase to another" [3]. The difference between various kinds of chromatography lies at two main levels (i) differences in the kinds of distribution systems used, particularly in terms of the phases employed and (ii) differences in the ways the phases are contracted or manipulated. There are four major kinds of chromatography:

1.1.1 Adsorption or Tswett Chromatography

It is founded on the differences in the adsorption coefficients of the solutes. In this the fixed (stationary) phase is solid e.g. alumina, magnesium oxides, silica gel etc. and the solutes are adsorbed in different parts of the adsorbent column. The adsorbed components are then eluted by passing suitable solvents (mobile phase) through the column. Column chromatography is a traditional sort of liquid chromatography and at a standstill because of its easiness of process and cheap natural history. It was utilized broadly for the taking out and refinement of the ecological and natural samples. The different sorts of sorbents were utilized chromatography i.e. silica, alumina, florisil etc. This modality has been reinstated by high pressure liquid chromatographic technology, which is supposed as better and advance developments.

1.1.2 Partition Chromatography

In partition chromatography, separation occurred due to the partition phenomenon of solute between stationary (liquid) and mobile phases. In paper chromatography fixed phase may be a liquid strongly adsorbed on a solid, which acts as a support. In this case, the solute gets distributed between the fixed liquid (stationary phase) and the moving liquid (solvent, mobile phase).

1.1.3 Ion Exchange Chromatography

Ion exchange chromatography involves the principle of ion exchange for the separation of compounds. Basically, it is useful for the resolution of ionic compounds. It may be of cation or anion exchange type. But sometimes, some adsorptive forces also contribute in the separation process.

1.1.4 Foam and Emulsion Chromatography

It utilizes the chemicals which form foam/micelles and the solutes are distributed between mobile phase and micelle. It is too termed as micellar chromatography. The surfactant in miceller chromatography contains a extended series hydrocarbon end and charged top. The arrangement of micelles happens in water mobile phase when the amount of the counter ions surpasses a serious micelle concentration. About 40 to 100 ions cumulative to shape approximately round particles with the hydrophobic end leaning towards the centre and the hydrophilic head oriented towards the exterior of the micellar particle. Therefore, a subsequent stage is fashioned and uncharged species may be solubilized into the micelles.

As seen above, all chromatographic processes involve a mobile phase, which passes over a stationary phase. Therefore, the solute is distributed between the two

phases and the particular reason for the type of distribution is the heart of the chromatographic system.

1.1.5 Thin Layer Chromatography

Thin layer chromatography (TLC) was first introduced by Izmailov and Sharaiber in 1938 [4]. Thin layer chromatography is often named by other names such as drop, strip, spread layer and surface chromatography. Due to its inexpensiveness, simplicity, sharpness of separation, high sensitivity, speed of separation and ease of recovery of separated compounds, thin layer chromatography has found increasing application in all branches of the analytical chemical science. This technique has been proved effective process for analytical investigations of those substances, which are available or present in very small or trace quantities. All chromatographic principles functioning in solid-liquid and liquid-liquid systems are also applicable to thin layer chromatography. Adsorption phenomenon has widely been used, but ion exchange and partition can also be applied in TLC. The choice of chromatographic principle is determined by the chemical nature of the compounds (to be resolved) and by the desired pattern of fractionation. Thin layer chromatography is considered to be more superior to column and paper chromatography because of following important reasons:

1. It requires less amount of substance.
2. It is less time consuming and takes 15-45 minutes for development.
3. Even strong acid can be safely sprayed on chromatoplates, if they are to be identified.

4. Capacity of adsorption in thin layer chromatography is higher than paper chromatography.

5. Corrosive mixture and standards can be run parallel.

A drop of solution containing the sample is spotted at the lower edge of the plate and allowed to develop. Migration then occurs as a result of flow by mobile phase, called the developer. Movement of developer is caused by capillary forces. The process of allowing the solvent to move along the chromatoplate is called development. The resolution is the degree of separation of components after the development. Loading is the amount applied as a spot or streak to the plate. The ratio of distance traveled by substance and mobile phase, both measured from the point of application of the sample, is termed as R_f (retention factor). The individual components can be characterized by R_f values, which can be calculated by the following formula:

R_f = Distance moved by the component/Distance moved by the solvent

1.1.6. High Performance Liquid Chromatography

HPLC as well recognized as high pressure liquid chromatography has made a significant contribution in the analysis of a variety of samples. Kirkland and Huber firstly introduced HPLC in 1964 [4]. In HPLC, a small diameter column (1-3 mm) with support particles sizes of 30 μm are used and the eluent is pumped through a column at a desired flow rate at high pressure. It has been found that separation by HPLC is possible about 100 times faster than any of conventional liquid chromatography. In comparison to classical column chromatography, where the columns are gravity fed and a separation can take hours and even days, HPLC can

provide analysis time of 5-30 minutes. HPLC is particularly suitable to the analysis of those compounds, which are not readily handled by other forms of chromatography. For example, thermally liable and polymeric compounds can be analyzed at ambient temperature by HPLC. Sometimes, sample clean-up is not required with HPLC and biological fluids can often be directly injected onto an HPLC column. Besides, much sample pretreatment can also be avoided because aqueous solvents can be used in HPLC. Because of all these advantages, HPLC has made a significant contribution in pharmaceutical, clinical, forensic and environmental analysis.

Thus, high performance liquid chromatography is a method of choice in separation science. In HPLC, the eluent from the solvent reservoir is filtered, pressurized and pumped through the chromatographic column. A mixture of solutes is injected at one end of the column, which gets separated into components on traveling through column. The separated solutes are monitored by detector and recorded as peaks on a chart recorder or by computer controlled software. The components of HPLC are shown in Figure 1.1.

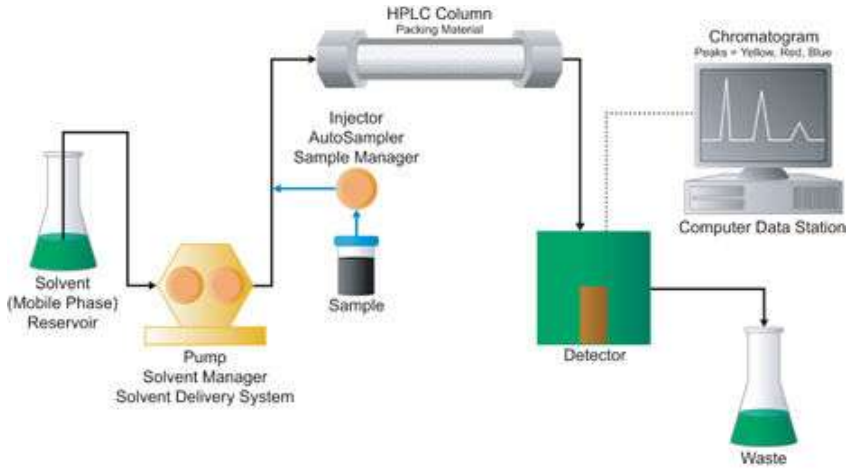

Figure 1.1: Components of HPLC.

The essential features of a modern high-pressure chromatograph are:

(a) Solvent delivery pump.

(b) A solvent delivery system, including pump.

(c) A means of introducing the sample, i.e. sample injection assembly.

(d) A chromatographic column.

(e) A detector and recording system.

In addition, on-line filters, pressure gauges, integrators, degasification unit and minor components may be required.

Some of the advantages of use of HPLC in brief are:

1. Separation fast and efficient (high resolving power).
2. Continuous monitoring of column effluent.
3. Separation and analysis of very complex mixtures.
4. Precise quantitative measures.
5. Recurring and reproducible analysis.
6. Mechanization of analytical procedure and data handling.
7. Excellent adsorption, partition, ion exchange and exclusion column separations.
8. Analysis of both aqueous and non-aqueous samples with little or no sample pretreatment.
9. Availability of a variety of solvents and column packing materials, providing an elevated selectivity for specific analysis.
10. Easy collection and isolation of separated components from the mobile phase for further analysis or characterization.

11. Completion of analysis in few minutes, with outstanding precision and accuracy.

The chromatographic divisions are distinguished by Retention (k), Separation (α), and Resolution (Rs) factors. The magnitudes can be ascertained by the following equations:

Retention factor (k) $= (t_r - t_0)/t_0$ …….....(1)

Separation factor (α) $= k_2/k_1$ …….....(2)

Resolution factor (Rs) $= \Delta t / ({}^1w_{1/2} + {}^2w_{1/2})$ ……... (3)

t_r and t_0; retention time and dead time of the column in minutes. Δt, ${}^1w_{1/2}$ and ${}^2w_{1/2}$ are the distinction of the retention times of two peaks, widths of peak 1 and peak 2 at half distance. If the personal magnitudes of α and Rs are 1.0 or larger, the separation is measured to be absolute. If personage magnitudes of the parameters are lesser than 1.0, the separation is measured to be imperfect.

No. of plates theoretical (N) distinguish the quality of column, larger the magnitude of N, more complex example mixture can be separated utilizing the column. The value of (N) can be pre-meditated by following equation:

$N = 16 (t_r / w)^2$ …….(4)

or $N = 5.54 [(t_r) / w_{1/2}]$ …….(5)

where t_r, w , and $w_{1/2}$ are the retention time in min. of the peak, peak width at base and half height of the peak. Retention time in equation 4 or 5 can be replaced by retention volume which means that the width is measured in volume units. Thus,

$N = 16 [V_R / w]^2$ ……..…….(6)

where, V_R is the retention volume (= retention time x flow rate of solvent).

Height equivalent to theoretical plate, HETP (h) is a part of a column, in which the mobile phase and the stationary phase are in equilibrium and may be determined by following equation:

$h = L / N$ ……(7)

L: length of the column.

1.1.7. Ultra Fast Liquid Chromatography

Ultra-Performance Liquid Chromatography (UFLC) was introduced in 2004 and it was a revolution in separation science. This instrument can be used conveniently for sub 2.0 micron particle size columns with augment in resolution, rate and feeling in liquid chromatography. First of all, a good advancement connecting concurrent innovation in particle skill and tool design was endeavored to gather and conquer the challenge of the separation science laboratory. This was carried out for making separation science scientists more winning and business more gainful and creative. For more than 40 years, dipping stationary phase element size has been browbeaten to get better chromatographic severance competence. UFLC technology had arrived at a upland in which the reimbursement of reducing element size could not be completely realize owing to the unhelpful power of equipment band dispersion and inadequate force variety. Theoretically, UFLC follows the van Deemter equation, which is an empirical formula showing the relationship between linear speed (flow rate) and plate height. The particle size is one of the parameters of the van Deemter equation, the curve produced may be utilized to examine chromatographic presentation. As the particle size reduces to fewer than 2.5 micro meters. There is a significant augment in

the efficiency with flow speeds are augmented or whilst linear velocities are amplified. The components of UFLC are shown in Figure 1.2.

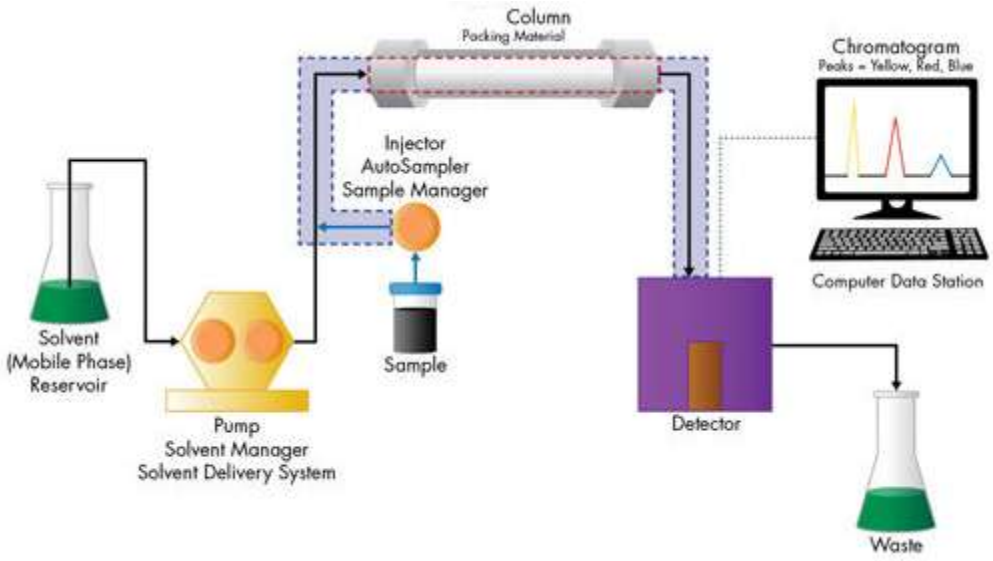

Figure 1.2: Components of Ultra Fast Liquid Chromatography (UFLC).

1.2. SAMPLE PREPARATION:

Biological samples contain various constituents, which may interfere during the course of analysis and, hence, sample grounding is an integral part of drugs and pharmaceutical analyses in water samples [5]. The sample preparation includes the sampling protocol, extraction of the required drug residue, concentration and purification.

1.2.1 Preservation

Occasionally, the laboratory and equipments are not accessible right away following sampling, particularly with the sampling is conceded out at distant areas. In such environment, the storeroom of samples is must. The conservation of the chemical individuality of the sample is main object of preservation. The assortment of the

conservation technique depends on the sort of sample, number, size and storeroom time, of samples. These water samples should be accumulated in an incubator at about 4ºC to avoid vanishing and decay of the drug residues. The samples having µg/L amounts should be estimated straight away. Occasionally, some preservatives should be utilized to accumulate the samples for drugs.

1.2.2 Extraction

The extraction is the next step to the sampling and many methods was used for the extraction purposes and mainly methods in this field are traditional having LLE, SPE and liquid chromatographic techniques. The taking out of biological samples is described briefly herein. Generally utilized methods for the extraction of drug residues from biological samples are liquid-liquid extraction and solid phase extraction but with the development in extraction science different kinds of chromatography modalities have also been used as the extraction methods. The chromatographic techniques used for this purpose are column chromatography (CC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), gel permeation chromatography (GPC) etc. The use of these modalities for the extraction of drug residues is given below in brief.

1.2.2.1 Solid Phase Extraction

Solid phase extraction method, produced by Zhang *et al.* **[6,7]**, is open from the problems of LLE and is fast, responsive with the revival of contaminants in the range of 90-95%. The utilization of SPE presents the compensation of handiness, price saving and negligible use of solvents **[8]**, and, therefore, approximately 60%

chromatographers are using this way for the sample handling. Furthermore, throughout the removal of the ecological samples, equally biogenic and anthropogenic molecules are taken out into organic and inorganic solvents. The composite matrix may interfere critically with the determination of the particular analytes. A variety of columns, disks and cartridges have been used for the extraction purposes but cartridges are most accepted taking out procedure [8]. The industrialists produced novel formats for conventional cartridges. Most SPE cartridges have medical grade polypropylene syringe barrels with porous PTFE or metal frit that contain 40 μm d_p packings. The cartridges give some benefits in comparison to disk as liquid flows earlier in cartridges in contrast to the disks. Mainly of the cartridges and disks are silica based polymeric packings.

The significant cartridges and disks are PL RD-S [9], HDG-C_{18} [10], RDS-$_{18}$ [11], C_{18} cartridge and SS-401, XAD-2 [12], C_{18} silica bonded cartridge [13], SEP-Pak C_{18} [14], extraction fibre [15], ODS impregnated polymers [16], C_8 disk [11] and C_{18} empore disk [17,18]. SPE engages the use of non-polar C_8 and C_{18} phases in the form of cartridges and disks. The disks are favoured to the cartridges, as the formers have elevated cross sectional area that gives some compensation, that are not probable in cartridges. In the disk, the reduced back pressure permits better flow rate as wide bed reduced the possibility of plugging. The embedding of the stationary phase into a disk stopped channelling and get better mass transfer [19]. Off-line or on-line SPE or solid phase micro extraction on a variety of types of silica bonded, polymeric or carbon sort phases were utilized for removal purpose [20]. Extraction efficiency in SPE was optimised and augmented by altering dissimilar parameters such

as ionic strength of the sample, elution, solvents, pH, elution gravity, and the contents of organic modifier in the sample, etc. Permissiveness of the method was recognized utilizing polymer membrane having the enmeshed sorbent particle in a web of polymer micro fibrils called membrane extraction disks. The parts of SPE are shown in Figure 1.3.

Figure 1.3: Components of solid phase extraction (SPE).

1.2.2.2 Liquid Chromatographic Extraction

Throughout current duration, liquid chromatography i.e. column chromatography (CC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and gel permeation chromatography (GPC) have received a wide spread attention for the extraction of inorganic and organic contaminants from the environmental samples. The reputation of the chromatographic techniques in the extraction of the pollutants is owing to lots of folds development of this technology, that can be utilized for the extraction of a wide range of contaminants. Furthermore, quick speediness and small use of the expensive solvents are the other property of chromatographic methods. The hyphenation of the chromatographic instrument with other analytical techniques has also put chromatographic modalities at its high horizons.

1.2.2.3 Clean-up

Biological samples have many previous matters as contamination and these got co-extracted with drug residues or metal ions of attention. Owing to the alike belongings of the co-extractives, they typically hinder in the analysis of the pollutants in almost every analytical methodology. Hence, clean-up procedure is needed merely for those samples, which are not clear. Chiefly samples of manufacturing and civic effluents need clean-up procedure. If clean-up is required, the recovery of each pollutant is extremely significant. It should be as possible as maximum 85% [21]. Clean-up of the extracted pollutants may be carried out by column chromatography, gel permeation chromatography, sweep co-distillation, liquid-liquid partition, cartridges and disks. On line SPE may be joined to membrane disk for the clean-up of

the pollutants species in water and other liquid biological samples. By means of a SFC/SPE methodology a better revivals of the drugs residues from water sample may be achieved.

1.2.2.4 Pre-concentration

Generally, the level of the drugs residues in the biological samples is below the recognition limits of the current detectors and, so, pre-concentration of the taken out (and cleaned) samples is needed. Generally, the size of the taken out solvent having pollutants is condensed to 0.5 to 1.0 mL. The traditional advance of vanishing of the solvents is utilized to focus the extracted contaminants. Though some strategy such as purge and tap device and cryogenically cooled capillary taps also was used for pre-concentration purpose [22]. Solvents with little boiling points are imagined as the best for pre-concentration end of view but the collection of the solvent depends on quite a few issues as by now argued. The great volume of the solvent obtained following removal is disappeared by distillation unit. Open vanishing of extracts on water bath may cause harsh dead of the contaminants and therefore, vanishing must be done at small temperature in compact pressure. Generally, rotary flash evaporator or Kuderna-Danish assembly is used for this reason.

Throughout extraction methods, particularly in LLE method, several unseen dampness may clearly remains in the samples along with organic solvents, that may make difficulty in the detection of the contaminates. So, dampness should be separated from the sample previous to its load on to the analytical equipment. Generally, the moisture from the organic solvent is separated by the adding of anhydrous sodium sulphate of elevated purity.

1.3. DRUGS:

The various class of drugs studied are cardiovascular, anti-diabetic and anti-histamines. These drugs were studied in human plasma.

1.3.1 Cardiovascular Drugs

Nowadays, about 33.3% populations in 194 countries are suffering from various cardiovascular diseases [23]. These ailments comprise angina pectoris, thyrotoxicosis, hypertension, migraine headaches cardiac arrhythmias, and glaucoma [24,25]. These diseases are due to abnormal changes in various biological activities. Therefore, the different drugs are used to cure cardiovascular diseases. For example, carvedilol and metoprolol are β-blockers relaxing blood arteries resulting into decrease in blood pressure. On the other hands, amiloride, hydrochlorothiazide and frusemide are diuretics reducing blood volume and decreasing blood pressure [26,27]. Losartan, olmesartan and telmisartan are angiotensin used to control blood pressure. Amlodipine is calcium channel blocker, used to low blood pressure and also to cure from angina pectoris. Sometimes, medicinal practioners prescribe the combination of three or more drugs. Besides, the combination treatment may lead a few side effects due to the creation of other molecules by interactions of these drugs in human body. The pharmacokinetics and pharmacodynamic of the reported drugs functions differently and vary in combination therapies. So, there is a huge demand to produce cardiac HPLC method, which can detect these drugs simultaneously. These drugs were mixed in human plasma and kept at 37ºC for 24 hrs to determine interaction products; if any. The cardiac drugs studied were amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, frusemide, telmisartan, losartan and olmesartan.

1.3.2 Anti-diabetic Drugs

The diabetes mellitus is a group of metabolic illnesses connecting high blood sugar. About 387 million people have been accounted diabetes worldwide in 2014 [28]. Also, 1.5 to 4.9 million deaths has been reported from 2012 to 2014. It was approximated that the figure of diabetic patients may augment up to 592 million by 2035. Hence, the planet economy is touching very much. The influence on financial system was approximated and establish to be 245 billion US $ in 2012 [29] and 612 billion US $ in 2014 in USA alone [30]. Owing to big number of the deaths and the financial stress, the different drugs are being utilized to manage the diabetes. The most important drugs are metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide. These drugs control diabetes by the different mechanisms. The studied drugs are metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide.

1.3.3 Anti-histamine Drugs

Antihistamines are drugs treat allergic rhinitis and other allergies. These drugs provide assistance to person having nasal overcrowding, sneezing, spots dust mites and allergy. Characteristically, persons receive antihistamines as a low-cost, general, over the oppose drug with little side effects. As an option to enchanting an antihistamine, persons endure from allergies can in its place keep away from the substance which irritates them. Antihistamines are, generally, small term treatment. The old and constant allergies augment the danger of physical condition trouble which antihistamines do not treat, counting sinusitis, asthma, and lower respiratory tract

illness. The studied drugs are phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine

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2.1. INTRODUCTION:

In this chapter the materials, methods and procedures, required to fulfill the necessity of the research work are explained. The detailed information of materials and methodologies used to separate, identify and quantify cardiac drugs, antidiabetic drugs and antihistamine drugs are given in this chapter. The materials include the details of suppliers of A.R. grade of these drugs and the reagents required to prepare and study sample preparation and Chromatographic behavior of the reported drugs. The methods describe the techniques used i.e. high performance liquid chromatography (HPLC) and solid phase extraction (SPE) analysis of cardiac drugs, antidiabetic drugs and antihistamine drugs. The necessary information regarding their application in monitoring the above mentioned cardiac drugs, antidiabetic drugs and antihistamine drugs are provided. The procedure applied for the collection of samples has been given in the following section along with other details.

2.2. PLASMA SAMPLES:

The first task is the sample preparation of the cardiac drugs, antidiabetic drugs and antihistamine drugs as these molecules are present in very minute level in the blood, so, this procedure requires a careful consideration. To simplify these complications, it is important to generate easy, speedy and trustworthy course of action for sample preparations. The method growth and system required for the utilize of supplies of recognized composition e.g. qualified suggested materials (internal standard). Consequently, spearing experimentation has to be carry out for excellence management of the technique. Under such type of cases stress has to be

put on the spiked measures as they apply a control on the revival magnitude. The sample grounding includes sample, removal, cleansing and pre-concentration phenomenon.

For the plasma sample compilation, sample approach comprises collection of Government/Private Blood Bank, sample bottle, volume compilation, sample management, carrying, conservation and storeroom. For the present thesis, plasma samples were collected from Government/Private Blood Bank. These plasma samples were preserved and stored at -5^{o}C for further use. An outline of the overall experimental setup is shown in Figure 2.1.

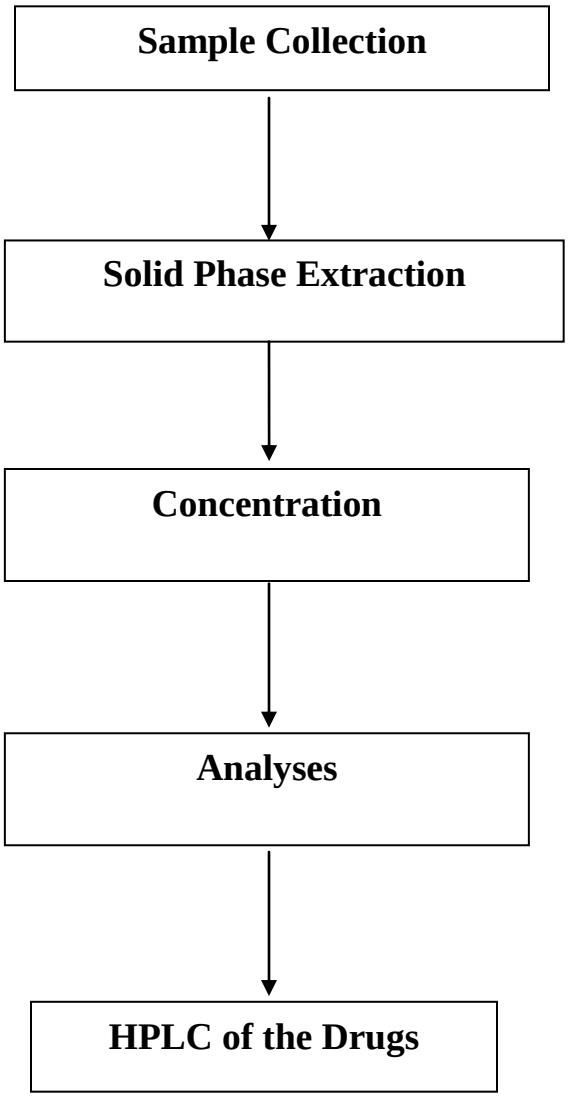

Figure 2.1: Flow diagram of the experimental setup.

2.3. ANALYSIS OF CARDIOVASCULAR DRUGS IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND SOLID PHASE EXTRACTION METHODS:

2.3.1 Experimental

2.3.1.1 Materials and Methods

The cardiac drugs (amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, fusemide, telmisertan, losartan and olmesartan) were obtained from different suppliers. The providers of these drugs were Anphar Laboratory, Gandhi Nagar, India (amiloride HCl), Sun Pharmaceuticals, Ahmed Nagar, India (metoprolol tartrate), CTX Life Science Pvt. Ltd., Surat, India (hydrochlorothiazide), Aurobindo Pharmaceuticals, Hyderabad, India (carvedilol), Prudence Pharmaceuticals, Ankleshwar, India (amlodipine besylate), Manglam Drug Organics, Mumbai, India (frusemide), Harika Drug Pvt. Ltd., Hyderabad, India (telmisartan), Vashudha Pharmaceuticals, Hyderabad, India (losartan potassium) and Nutra Specialities Pvt. Ltd., Bharathi Nagar, India (olmesartan). Acetonitrile and triethyl amine of AR grade were purchased from Merck, India. . Disodium hydrogenphosphate ($Na_2HPO_4.2H_2O$) of AR grade and ortho phosphoric acids were purchased from Merck, Mumbai, India. Fresh Frozen Human Plasma (Mfg. License No. 504) was collected from Rotary Blood Bank, New Delhi, India. Millipore water was prepared using a Millipore Milli-Q (Bedford, M.A., U.S.A.). pH meter was used for adjusting mobile phase.

The standard solutions of individual (1.0 mg mL^{-1}) and the mixture (0.0001-0.025 $mgmL^{-1}$) of these molecules were made in methanol. The store solutions were sheltered commencing beam sample vials by casing with aluminium thwart

and stock up at 4ºC. A pipettes and 10.0 mL volumetric flagon were utilized for sequential dilutions of the molecules to get the necessary concentration array (0.001-0.025 mg mL^{-1}).

Purified water was ready by Millipore Milli-Q (Bedford, M.A., U.S.A.). Acetone, acetonitrile, acetic acid, methanol, and sodium di-hydrogen phosphates (A.R. grade) were bought from Merck, Mumbai, India. Na_2HPO_4 was bought from Fisher Scientific Company, USA. pH was recorded by utilizing a pH meter (Hach, Loveland Co.). SPE was conceded out by utilizing C_{18} Sep-Pak Vac (1.0 mL) sealed unit, which was bough from Waters, USA.

2.3.1.2 Instruments Used

HPLC system was of Shimadzu (Kyoto, Japan) consisting of system controller pump (model: SCL-10AVP), liquid chromatograph (model: LC-10ATVP) and CLASS VP software, with UV-Vis detector (model: SPD-10A). SunShell C_8 column (150 x 4.6mm, 2.6 µm) of Chromanik Japan was used for this purpose.

2.3.2 Methodology

2.3.2.1 Preparation of Standard Solutions

The normal solutions of entity (1.0 $mgmL^{-1}$) and the mixture (0.0001-0.025 $mgmL^{-1}$) of these molecules were prepared in methanol. The store solutions were sheltered from beam sample vials by wrapper with aluminium thwart and store up at 4ºC. A pipettes and 10.0 mL volumetric flagon were used for sequential dilutions of these molecules to get the necessary concentration choices (0.001-0.025 $mgmL^{-1}$).

2.3.2.2 Extraction of the drugs from plasma by Solid Phase Extraction

Solid phase extraction of these drugs was conceded as per the normal measures [1-4]. To decide the generation of any new molecule in the human body during combination therapy, these drugs were mixed individually and in mixture with human plasma. 1.0 mL (1.0 $mgmL^{-1}$) of these drugs was assorted with 5.0 mL fresh frozen plasma individually and their mixture, separately and respectively. These samples were reserved in an incubator for 37ºC for 24 hrs. For extraction purpose, acetone (15.0 mL) was assorted with every sample vial and reserved for 30 min. The samples were centrifuged at 10,000 rpm (11,180 g) for 10.0 min to divide the supernatant. The supernatant was disappeared to aridness in vacuum. The remains was re-dissolved in 10.0 mL phosphate buffer (20 mM, pH 7.0), respectively. Sep-Pac C_{18} cartridges were pre-conditioned with 2.0 mL MeOH and 5.0 mL Millipore H_2O. The buffers containing individual and their mixture drugs were conceded through the cartridges with 0.1 $mLmin^{-1}$ flow rate, persuaded by cartridges cleaning with 2.0 mL Millipore water at 0.1 $mLmin^{-1}$ flow rates. Further, the cartridges were dried out by passing scorching air. The elution of the described drugs was done using 10.0 mL methanol at 0.1 $mLmin^{-1}$ flow rate. The eluted MeOH with cardiovascular drugs were intensed in vacuum to 0.5 mL, separately and respectively. The samples were used for HPLC studies.

2.3.2.3 HPLC Conditions

Whole the experimentation was carried out by HPLC system as mentioned on top of. The volume of 5.0 micro litters of model solutions of each drug and their combination (0.005 $mgmL^{-1}$ of each drug in MeOH) were loaded on HPLC

instrument. The eluent used was phosphate buffer (65% with 0.8% TEA) in acetonitrile with flow rate of 0.5 $mLmin^{-1}$ under isocratic mode. UV detection was achieved at 230 nm with temperature at 40±1ºC. Fresh mobile phase was prepared, clean and degassed daily prior to use. The chromatographic variable like retention (k), separation (α) and resolution (R_s) factors for the reported drugs were ascertained. The command of eluted peaks was ascertained by operating each entity drug. The qualitative and quantitative analyses were optimized by considering retention times and peak areas, respectively. The number of hypothetical plates and shadowing variables were also calculated. The HPLC method was optimized and authenticated. The developed HPLC method was practical for analyses of the drugs in plasma samples.

2.3.3 Validation

HPLC method was authenticated by calculating different HPLC variables [5-10]. For method validation linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, specificity, ruggedness and robustness were ascertained. The LOD and LOQ were ascertained by inserting lowest concentration of these drugs. The outcome of the numerical analysis of the experimentation of HPLC data like correlation coefficients (CC), relative standard deviation (SD), and confidence limits (CL) were ascertained by Microsoft Excel software program. Excellent linearity of the calibration plots and the insignificant disperse of experimentation positions were utilized for ascertaining relative standard deviations and correlation constant. Method robustness was determined by adaptability of the experimentation variables, which affected the peak areas.

2.3.3.1 Linearity

The linearity of this method was confirmed by least squares linear regression analysis of calibration plot. Besides, the linearities of calibration plots (peak area versus amount) for amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan standards were optimized and evaluated by varied amount assortments. Equivalent volume (5.0 µL) of the typical as explained over was laden onto HPLC equipment. The HPLC chromatograms of the statement drugs were developed separately and respectively. The calibration curves of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisertan, losartan and olmesartan were built utilizing the seen peak areas versus ostensible amounts of the described drugs.

2.3.3.2 Detection and Quantitation Limits

The detection and quantitation limits for the described drugs were ascertained as three and five epochs to the base line noise, respectively. As per the United States Pharmacopoeia this standard was followed.

2.3.3.3 Specificity

The specificity of the process was ascertained by seeing whichever intrusion in HPLC results owing to the presence of a few adulterations in the benchmark samples. The benchmark models were diverged with very low quantity of crude reported drugs to create it contaminated.

2.3.3.4 Precision

Precision data was calculated at 3 dissimilar quantities i.e. 0.001, 0.005 and 0.025 $mgmL^{-1}$ of all the reported drugs. Five rests of HPLC experiments were done for all these three quantities.

2.3.3.5 Accuracy

Accuracy of HPLC was determined by dissimilar quantities of the described molecules. Three concentrations utilized were 0.001, 0.005 and 0.025 $mgmL^{-1}$. The HPLC runs were optimized five times (n = 5). The accuracies were ascertained by interpolation of five replicates peak areas of these reported drugs.

2.3.3.6 Robustness

Robustness was ascertained by changing a minor deviation in the chromatographic experimental conditions like flow rate, temperature, eluent constituents and λmax. The peak area and shape and retention time were analyzed in the well-known and a little diverged experimentation circumstances.

2.3.3.7 Ruggedness

Ruggedness of the method was ascertained by changing the experimentation conditions like several handling person and dissimilar times.

2.3.4 Quantitative Analysis

The quantitative analysis of the drugs was done by customary technique of assessment [11-13]. The quantitative determination of the drugs was ascertained by contrasting the peak areas of the drugs in customary and plasma samples. Through dissimilar quantities of the drugs the limit of detection and limit of quantification were ascertained. The outcomes of the arithmetical analysis of the

experimentation data like the normal correlation coefficient deviation, and confidence levels were ascertained by Microsoft Excel software program. The subsequent equation was utilized to calculate the quantities of the drugs.

Concentration of the drugs = $[C_{std} \times A_{std}]/A_{ss}$
in plasma sample.

where,

C_{std} : Conc. of typical solution

A_{ss} : Peak area of sample

A_{std} : Peak area of normal

The percentage recoveries of the drugs were ascertained by standardizing the technique using tyrosine as an interior standard. The error of the method was also ascertained by using tyrosine. The percentage recoveries of the drugs are ascertained by contrasting the quantity spiked in plasma and the quantity achieved following HPLC study.

2.4. ANALYSIS OF ANTI-DIABETIC DRUGS BY ULTRA FAST LIQUID CHROMATOGRAPHY AND SOLID PHASE EXTRACTION METHODS:

2.4.1 Experimental

2.4.1.1 Materials and Methods

The standards of metformin HCl (Aarti Drug Limited, Patana, Udhargali, India), vildagliptin, linagliptin, gliclazide, sitagliptin, (Nutra Specialities Pvt. Ltd., Bharathi Nagar, India), glimepiride (Aarti Drug Limited, Patana, Udhargali, India), pioglitazone (Indoco remedies, Mumbai, India) and repaglinide (Dr. Reddy's Laboratories, India). ACN, MeOH and $CH_3CH_2NH_2$ of HPLC grade

were given by Merck, Bombay, India. NH_4COOCH_3 and *o*-H_3PO_4 of A.R. grade were given by Merck, Bombay, India. Fresh Frozen Human Plasma (Mfg. License No. 504) was given by Rotary Blood Bank, New Delhi, India. Millipore water was prepared by Millipore Milli-Q (Bedford, M.A., USA).

2.4.1.2 Instruments Used

UFLC system used was of Shimadzu (Kyoto, Japan) having pump (model: SCL-10AVP), liquid chromatograph (model: LC-10ATVP) and CLASS VP software, with UV-Vis. detector (model: SPD-10A). Sunshell C_{18} column (150 x 4.6 mm, 2.6 µm) was supplied by Chromanik, Japan. Sep-Pac C_{18} cartridges were purchased from Waters, USA. The cartridge contains 50 mg sorbent (55-105 µm particle size). Sep-Pac C_{18} cartridges were purchased from Waters, USA.

2.4.2 Methodology

2.4.2.1 Preparation of the Standard Solutions

The normal solutions of entity (1.0 $mgmL^{-1}$) and the mixture (0.0001-0.025 $mgmL^{-1}$) of these molecules were prepared in eluent. The store solutions were sheltered from beam sample vials by wrapper with aluminium thwart and store up at 4ºC. A pipettes and 10.0 mL volumetric flagon were used for sequential dilutions of these molecules to get the necessary concentration choices.

2.4.2.2 Extraction of the drugs from plasma by Solid Phase Extraction

The described drugs were diverged with plasma independently and in grouping. 1.0 mL (1.0 $mgmL^{-1}$) of these drugs was thorned with 5.0 mL human plasma independently and their combination, unconnectedly and correspondingly.

The assorted plasma samples were reserved in an incubator for 37 ºC for 24 hrs. 15.0 mL CH_3COCH_3 was assorted to every sample vial and reserved for 30 minutes. These were centrifuged at 10,000 rpm (11,180 g) for 10.0 min to get rid of the supernatant. The supernatant was vanished in vacuum and stay behind was re-dissolved in 10.0 mL phosphate buffer (25 mM, pH 7.0), correspondingly. Sep-Pac C_{18} cartridges of 1.0 mL were pre-conditioned using MeOH (2.0 mL) and Millipore water (5.0 mL), independently and correspondingly. The buffers having individual and assortment of the drugs were flowed via the cartridges at 0.1 $mLmin^{-1}$ flow rate; tracking by cartridges washing with Millipore (2.0 mL) water at 0.1 $mLmin^{-1}$ flow rates. In addition, these cartridges were dried by flowing scorching air. The elution of the reported drugs was completed by 10.0 mL methanol at 0.1 $mLmin^{-1}$ flow rate. The eluted MEOH solutions with these drugs were intensed in vacuum to 0.5 mL, independently and correspondingly. These samples were used for UFLC analyses.

2.4.2.3 UFLC Conditions

The experimentation was done by UFLC system as described above. The aliquots of 5.0 µL of the benchmark solutions of every drug and the combination were loaded on UFLC instrument, independently and correspondingly. The eluent used was acetate buffer (0.05% TEA in 0.05 M ammonium acetate of pH 7.0 with H3PO4)-acetonitrile (60:40, v/v). Acetate buffer of pH 7.0 (for mobile phase) was made by weighing 3.08 g NH_4COOCH_3 (0.05 M, Mol. Wt. 77.08) in conical flask and adding 800 mL water and 0.4 mL $CH_3CH_2NH_2$. It was sonicated for 5 minutes. pH was continued until 7.0 with *o*-phosphoric acid. The eluent was ready

by adding 300 mL acetate buffer solution to 200 mL ACN. It was filtered and degassed every day previous to use. The flow rate was 1.0 $mLmin^{-1}$ with detection at 210 nm and temp. at 45 ± 1ºC. UFLC parameters viz. retention (k), separation (α) and resolution (Rs) factors for the reported drugs were ascertained. The command of the eluted peaks was ascertained by doing UFLC experimentation with every being drug. The qualitative and quantitative estimation were optimized by retention times and peak areas, correspondingly. UFLC method was optimized and validated. The developed and validated UFLC method was utilized for the analyses of these drugs in human plasma samples.

2.4.3 Validation

UFLC method was validated by manipulating different UFLC parameters [5-10]. The linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, specificity, robustness, accuracy and ruggedness were ascertained. The LOD and LOQ were determined using minimum concentration of these drugs. The statistical analyses of the experimental data viz. relative standard deviation (SD), correlation coefficients (CC) and confidence limits (CL) were determined by Microsoft Excel software program. Good linearity of the calibration plots and the small scatter of the experimental points were used for determining the correlation coefficients and relative standard deviations. The robustness of the method was ascertained by versatility of the experimental factors that affected the peak areas.

2.4.3.1 Linearity

The linearity of UFLC method was confirmed by least squares linear regression analysis of the calibration plot. Besides, the linearities of calibration plots (peak area versus amount) for metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide standards were optimized and compared by different concentration ranges. Equivalent volume of 5.0 µL of the typical as explained over was loaded on UFLC. UFLC chromatograms of the statement drugs were developed independently and correspondingly. The calibration curves of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were built utilizing the seen peak areas versus ostensible amounts of the described drugs.

2.4.3.2 Detection and Quantitation Limits

The detection and quantitation limits for the described drugs were ascertained as three and five epochs to the base line noise, correspondingly. United States Pharmacopoeia standards were followed for the purpose [14].

2.4.3.3 Specificity

The specificity of the process was ascertained by seeing whichever intrusion in HPLC results owing to the presence of a few adulterations in the benchmark samples. The benchmark models were diverged with very low quantity of crude reported drugs to create it contaminated.

2.4.3.4 Precision

Precision data was calculated at 3 dissimilar quantities i.e concentrations *i.e.* 0.04, 0.05 and 0.06 $mgmL^{-1}$ of all the reported drugs. Five rests of HPLC experiments were done for all these three quantities.

2.4.3.5 Accuracy

Accuracy of HPLC was determined by dissimilar quantities of the described molecules. Three concentrations utilized were 0.04, 0.05 and 0.06 $mgmL^{-1}$. The HPLC runs were optimized five times (n = 5). The accuracies were ascertained by interpolation of five replicates peak areas of these reported drugs.

2.4.3.6 Robustness

Robustness was ascertained by changing a minor deviation in the chromatographic experimental conditions like flow rate, temperature, eluent constituents and λmax. The peak area and shape and retention time were analyzed in the well-known and a little diverged experimentation circumstances.

2.4.3.7 Ruggedness

Ruggedness of the method was ascertained by changing the experimentation conditions like several handling person and dissimilar times.

2.4.4.8 Quantitative Analysis

The quantitative analysis of the drugs was done by customary technique of assessment [11-13]. The quantitative determination of the drugs was ascertained by contrasting the peak areas of the drugs in customary and plasma samples. Through dissimilar quantities of the drugs the limit of detection and limit of quantification were ascertained. The outcomes of the arithmetical analysis of the

experimentation data like the normal correlation coefficient deviation, and confidence levels were ascertained by Microsoft Excel software program. The subsequent equation was utilized to calculate the quantities of the drugs.

Concentration of the drugs in plasma sample. = $[C_{std} \times A_{std}]/A_{ss}$

where,

C_{std} : Conc. of typical solution

A_{ss} : Peak area of sample

A_{std} : Peak area of normal

The percentage recoveries of the drugs were ascertained by standardizing the technique using tyrosine as an interior standard. The error of the method was also ascertained by using tyrosine. The percentage recoveries of the drugs are ascertained by contrasting the quantity spiked in plasma and the quantity achieved following UFLC study.

2.5. ANALYSIS OF ANTI-HISTAMINE DRUGS BY ULTRA FAST LIQUID CHROMATOGRAPHY AND SOLID PHASE EXTRACTION METHODS:

2.5.1 Experimental

2.5.1.1 Materials and Methods

The standards of anti-histamine drugs (phenylephrine HCl, cetirizine HCl, loratadine HCl, montelukast sodium and ebastine) were purchased from different manufacturers. The suppliers of these drugs were Divis Laboratories, Hyderabad, India, Vaikunth Chem, P. Ltd, Ankleshwar, Gujarat, India, Bashudha Pharma Chem. Limited, Hyderabad, India, Metrochem API Pvt. Ltd., Hyderabad, India

and R.L. Fine Chem., Bangalore, India, respectively. ACN, EtOH and $CH_3CH_2NH_2$ of HPLC grade were purchased from Merck, Bombay, India. NH_4COOCH_3 and *o*-H_3PO_4 of A.R. grade were given by Merck, Bombay, India. Fresh Frozen Human Plasma (Mfg. License No. 504) was given by Rotary Blood Bank, New Delhi, India. Millipore water was made by Millipore Milli-Q (Bedford, M.A., USA).

2.5.1.2 Instruments Used

UFLC system used was of Shimadzu (Kyoto, Japan) having pump (model: SCL-10AVP), liquid chromatograph (model: LC-10ATVP) and CLASS VP software, with UV-Vis. detector (model: SPD-10A). Sunshell C_{18} column (150 x 4.6 mm, 2.6 µm) was supplied by Chromanik, Japan. Sep-Pac C_{18} cartridges were purchased from Waters, USA. The cartridge contains 50 mg sorbent (55-105 µm particle size). Sep-Pac C_{18} cartridges were purchased from Waters, USA.

2.5.2 Methodology

2.5.2.1 Preparation of the Standard Solutions

The standard solutions of the individual and their mixture were prepared in mobile phase. Further final dilution for Phenylephrine HCl, Cetirizine HCl, Loratadine HCl, Montelukast sodium and Ebastine were carried out using the mobile phase to make final concentration of 0.01624 $mgmL^{-1}$, 0.007696 $mgmL^{-1}$, 0.007852 $mgmL^{-1}$, 0.015908 $mgmL^{-1}$ and 0.008044 $mgmL^{-1}$ respectively. The stock solutions were protected from light by covering with aluminium foil and stored at 4º C.

2.5.2.2 Extraction of the drugs from plasma by Solid Phase Extraction

The described drugs were assorted with plasma independently and in amalgamation. 1.0 mL (1.0 $mgmL^{-1}$) of these drugs was thorned with 5.0 mL human plasma independently and their mixture, discretely and correspondingly. The mixed plasma samples were reserved in an incubator for 37 ºC for 24 hrs. 15.0 mL CH_3COCH_3 was assorted to every sample vial and reserved for 30 minutes. These were centrifuged at 10,000 rpm (11,180 g) for 10.0 min to take away the supernatant. The supernatant was disappeared in vacuum and stay put was re-dissolved in 10.0 mL phosphate buffer (25 mM, pH 7.0), correspondingly. Sep-Pac C_{18} cartridges of 1.0 mL were pre-conditioned using MeOH (2.0 mL) and Millipore water (5.0 mL), independently and correspondingly. The buffers having entity and mixture of the drugs were passed via the cartridges at 0.1 $mLmin^{-1}$ flow rate; go after by cartridges washing with Millipore (2.0 mL) water at 0.1 $mLmin^{-1}$ flow rates. In addition, these cartridges were dried by flowing scorching air. The elution of the reported drugs was done by 10.0 mL MeOH at 0.1 $mLmin^{-1}$ flow rate. The eluted MeOH solutions having these drugs were concentrated in vacuum to 0.5 mL, independently and correspondingly. These samples were used for UFLC analyses.

2.5.2.3 UFLC Conditions

The experiments were done by UFLC system as described above. The aliquots of 5.0 µL of the standard solutions of each drug and the mixture were loaded onto UFLC instrument, independently and correspondingly. The mobile phases used were-

0.01M ammonium acetate pH 5.0: Acetonitrile: ethanol (25:30:45).

1. Preparation of 0.01M ammonium acetate: weigh 77.08 mg of ammonium acetate and dissolve in 100 mL of water. Adjust pH 5.0 with glacial acetic acid.
2. Mix 25 mL of ammonium acetate buffer pH 5.0 with 30 mL of acetonitrile and 45 mL of ethanol.
3. Filter the mobile phase through 0.2 micron membrane filter.

It was filtered and degassed daily prior to use. The flow rate was 1.0 mL min^{-1} with detection at 210 nm and temperature at 35±1 ºC. UFLC parameters *viz.* retention (k), separation (α) and resolution (R_s) factors for the reported drugs were calculated. The order of the eluted peaks was determined by doing UFLC experiments with each individual drug. The qualitative and quantitative analyses were optimized by retention times and peak areas, correspondingly. UFLC method was optimized and validated. The developed and validated UFLC method was applied for the analyses of these drugs in human plasma samples.

2.5.3 Validation

UFLC method was validated by manipulating different UFLC parameters from second paper [15-17]. For method validation linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, specificity, ruggedness and robustness were ascertained. The LOD and LOQ were ascertained by inserting lowest concentration of these drugs. The outcome of the numerical analysis of the experimentation of HPLC data like correlation coefficients (CC), relative standard deviation (SD), and confidence limits (CL) were ascertained by Microsoft Excel software program. Excellent linearity of the calibration plots and

the insignificant disperse of experimentation positions were utilized for ascertaining relative standard deviations and correlation constant. Method robustness was determined by adaptability of the experimentation variables, which affected the peak areas.

2.5.3.1 Linearity

The linearity of UFLC method was determined by least squares linear regression analysis of the calibration curve. The linearity of the calibration curves (peak area *vs.* concentration) for phenylephrine HCl, cetirizine HCl, loratadine, montelucast sodium and ebastine standards were optimized and compared by different concentration ranges. Equal amount of 5.0 µL of the standards as described above was loaded onto UFLC. UFLC chromatograms of the reported drugs were obtained independently and correspondingly. The calibration curves of Phenylephrine HCl, Cetirizine HCl, Loratadine, Montelucast sodium and Ebastine were developed by the observed peak areas *vs.* nominal concentrations of these molecules.

2.5.3.2 Detection and Quantitation Limits

The limits of detection (LOD) and quantitation (LOQ) for these drugs were ascertained as three and ten times to the baseline noise, correspondingly. United States Pharmacopoeia standards were followed for the purpose [8].

2.5.3.3 Specificity

The specificity of the process was ascertained by seeing whichever intrusion in UFLC results owing to the presence of a few adulterations in the benchmark

samples. The benchmark models were diverged with very low quantity of crude reported drugs to create it contaminated.

2.5.3.4 Precision

The precision data was calculated at three different concentrations *i.e.* 0.04, 0.05 and 0.06 $mgmL^{-1}$ of all the reported drugs. Three experiments of UFLC were done for all these three quantities.

2.5.3.5 Accuracy

Accuracy of UFLC method was ascertained by various concentrations of the reported drugs. Three concentrations used were 0.04, 0.05 and 0.06 $mgmL^{-1}$. The UFLC experiments were optimized five times (n = 5). The accuracies were ascertained by interpolation of 5 replicates peak areas of these drugs.

2.5.3.6 Robustness

Robustness was ascertained by changing a minor deviation in the chromatographic experimental conditions like flow rate, temperature, eluent constituents and λmax. The peak area and shape and retention time were analyzed in the well-known and a little diverged experimentation circumstances.

2.5.3.7 Ruggedness

Ruggedness of the method was ascertained by changing the experimentation conditions like several handling person and dissimilar times.

2.5.4 Quantitative Analysis

The quantitative analysis of phenylephrine HCl, cetirizine HCl, loratidine HCl, montelukast sodium and ebastine drugs was carried out by usual method of comparison [11-13]. The quantitative ascertaining of phenylephrine HCl,

cetirizine HCL, loratidine HCl, montelukast sodium and ebastine drugs was ascertained by contrasting the peak areas of anti-histamine drugs in normal and that in the reaction mixture. By using different concentrations anti-histamine drugs, the detection and limit of quantification limits were ascertained. The outcomes of the arithmetical analysis of the experimentation data like the correlation coefficient, standard deviation, and confidence levels were ascertained by Microsoft Excel software program. The subsequent equation was utilized to calculate the quantities of anti-histamine drugs.

Concentration of anti-histamine drugs in reaction mixture. = $[C_{std} \times A_{std}]/A_{ss}$

where,

C_{std} : Conc. of standard solution

A_{std} : Peak area of standard

A_{ss} : Peak area of sample

The percentage recoveries of anti-histamine drugs were ascertained by standardizing the technique using an interior standard. The percentage recoveries of anti-histamine drugs were ascertained by contrasting the quantities spiked in distilled water and the quantities achieved after UFLC analysis.

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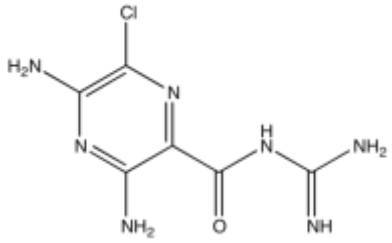
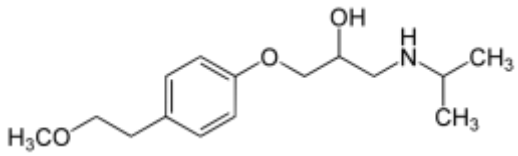
3.1. INTRODUCTION:

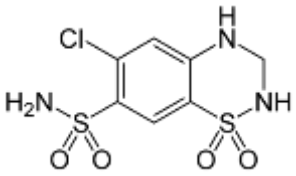
Nowadays, about 33.3% populations in 194 countries are suffering from various cardiovascular diseases [1]. These ailments comprise hypertension, angina pectoris, cardiac arrhythmias, thyrotoxicosis, migraine headaches and glaucoma [2,3]. These diseases are due to abnormal changes in various biological activities. Therefore, the different drugs are used to cure cardiovascular diseases. For example, carvedilol and metoprolol are β-blockers relaxing blood arteries resulting into decrease in blood pressure [4]. On the other hands, amiloride HCl, hydrochlorothiazide and frusemide are diuretics reducing blood volume and decreasing blood pressure [5,6]. Losartan, olmesartan and telmisartan are angiotensin used to control blood pressure [7]. Amlodipine is calcium channel blocker, used to low blood pressure and also to cure from angina pectoris [8]. The structures of these drugs are shown in Figure 3.1. Sometimes, medicinal practioners prescribe the combination of three or more drugs. Besides, the combination therapy may lead some side effects due to the formation of other molecules by interactions of these drugs in human body. The pharmacokinetics and pharmacodynamic of the reported drugs functions differently and vary in combination therapies. Therefore, there is a great need to develop cardiac HPLC method, which can detect these drugs simultaneously. These drugs were mixed in human plasma and kept at 37ºC for 24 hrs to determine interaction products; if any.



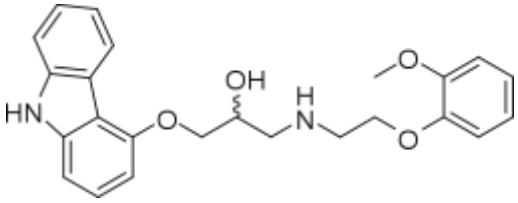
Amiloride



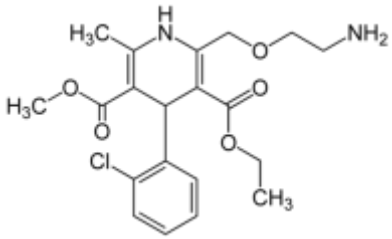
Metoprolol



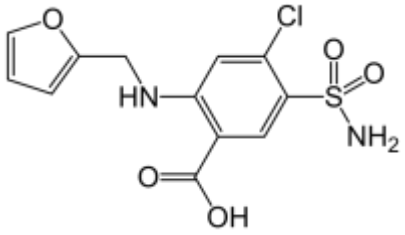
Hydrochlorothiazide



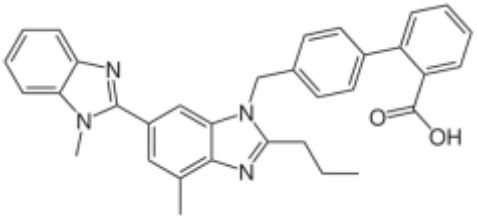
Carvedilol



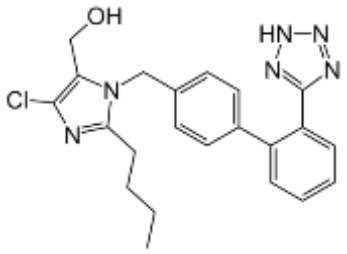
Amlodipine



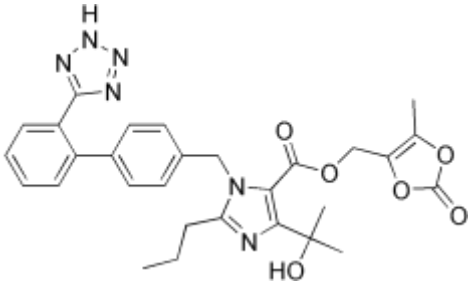
Frusemide



Telmisartan



Losartan



Olmesartan

Figure 3.1: Chemical structure of cardiovascular drugs.

3.2. LITERATURE SURVEY:

Analysis includes the HPLC methodology used to identify cardio vascular drugs. These analysis and separation are done by taking the advantage of difference in their properties [9]. The separation and identification of cardio vascular drugs are carried by extraction and separation techniques. The most commonly used techniques are solid phase extraction (SPE) and high performance liquid chromatography (HPLC). A thorough search of literature on the analyses of cardio vascular drugs was carried out through Scifinder, Scopus, and Science direct and peer reviewed Journals. This is discussed in the following sub-sections.

3.2.1. Amiloride HCl

The trade name of amiloride HCl is Midamor and is a medicine used with other medications to treat high blood pressure or swelling owing to heart failure or cirrhosis of the liver [10]. Amiloride HCl was prepared in 1967 and is the most effectual and safe drug as per World Health Organization's List of Essential Medicines, [11].

Amiloride HCl is frequently utilized with a thiazide or loop diuretic. Onset of accomplishment is about two hours and it ends for about a day. The common side effects are vomiting, loss of appetite, rash, high headache and blood potassium. The risk of high blood potassium is superior in those with diabetes, kidney problems, and olders. This drug is in the potassium sparing diuretic family of medicines. It acts by raising the quantity of sodium and lessening the quantity of potassium freed by the distal tubule of the kidney. Therefore, the analysis of amiloride is required in plasma, urine and other biological systems. Some papers

describe the analysis of this drug and are summarized below. Youssef et al. [12] accounted HPLC technique for analysis of amiloride hydrochloride in combined dosage forms. An RP-HPLC separation of the cited drug was done on an RP stainless steel C_{18} stationary phase (250 x 4.6 mm id) with a gradient elution system of methanol and 0.05 M aqueous phosphate buffer adjusted to pH 4 as the mobile phase, at the flow rate of 1.0 $mLmin^{-1}$. The quantitation was achieved with photodiode array detection at 275 nm for at 225 nm. The calibration graphs for each drug were rectilinear in the range of 2-50, 25-150, and 2-100 μgmL^{-1}. The planned chromatographic method was effectively utilized for the estimation of the investigated drug in pharmaceutical preparations. Nagori and Solanki [13] used HPLC for analysis of amiloride hydrochloride in tablet using HIQ SIL, C_{18} (250 × 4.6 mm, 5 µm) column with 50 mM phosphate buffer:ACN (50:50 v/v, pH 3.0) eluent, 1.0 $mLmin^{-1}$ flow and 283 nm detection. The retention time for for amiloride hydrochloride was 10.002 min. Amiloride hydrochloride showed a linear response in the concentration range of 10-100 μgmL^{-1}. The mean recoveries found for amiloride hydrochloride was 100.09%. The developed technique was established to be simple, accurate, precise and selective for simultaneous estimation of amiloride hydrochloride in tablets. Elshanawane et al. [14] described an HPLC technique with photometric detection for amiloride hydrochloride in human urine using chlorthalidone as the internal standard. A cyanopropyl column with the 10 mM KH_2PO_4 solution (pH 4.5)-methanol (70 + 30, v/v) as eluent and 1.0 $mLmin^{-1}$ flow. The technique was authenticated in terms of correctness, exactitude, absolute recovery, freeze-thaw stability, bench-top stability, and re-injection reproducibility.

The procedure shows good accuracy, repeatability, and selectivity. Moreover, the method was applied directly to urine that had not undergone prior treatment. The method, which is rapid, simple, and does not require any separation steps, has been successfully applied to the assay of human urine containing amiloride hydrochloride. El-Gindy et al. [15] used HPLC for amiloride hydrochloride using C_{18} column with ACN-5mM heptansulphonic acid sodium salt (20:80, v/v, pH 4.4) as eluent and 274 nm detection.

Zecevic et al. [16] reported HPLC having C_{18} column and 280 nm detection for amiloride. The eluent was water:methanol (60:40) and pH 3.2 (pH adjusted to 3.2 with CH_3COOH). Furthermore, the same group [17] applied a multi-factor optimization method to develop an HPLC method for amiloride with C_{18} column with detection at 286 nm. The mobile phase composition provided amiloride after optimization. Kartal and Erk [18] used accurate, rapid, specific, and precise HPLC for amiloride in tablet. Column was LiChrosorb RP-C_{18} with 0.025 M ortho-phosphoric acid (adjusted to pH 3.0 with $CH_3CH_2NH_2$-ACN (84:16 v/v) as eluent, 1.2 $mLmin^{-1}$ flow and 278 nm detection. Lee and Tannock I. [19] carried out kinetics of amiloride and analogs using C_{18} column and mobile phases as: (A): 100% CAN and (B): 0.15 M H_3ClO_4 with 1.2 $mLmin^{-1}$ flow and 365 nm detection. The system was run under non linear gradient mode with 0.1-50 µM linearity range.

Zivanovic et al. [20] carried out an HPLC for amiloride on C_{18} column with 286 mm detection. The consequence of concurrently changeable pH and MeOH to H_2O ratio in the eluent was studied for the purpose. The method was utilized for

quantitative analysis of tablet. The tablets were taken out with MeOH having phenacetin (as the internal standard). Selinger [21] performed an HPLC for amiloride in airway surface. The system comprised Zorbax column with 25% ACN in 0.05 M phosphate buffer and 360/420 nm fluorescence detection. The internal standard was triamterene. Recovery of amiloride was non linear, most likely owing to a sorption phenomenon. Accurateness of the method at low quantity might be pretentious by the option of filter paper and the attendance of endogenous plasma workings. Alliegro et al. [22] developed an HPLC method for amiloride in plasma samples on C_8 column using PLC 6% ACN − 45% MeOH − 5.4% CH_3COOH, adjusted to pH 4.0 with CH_3NH_2. The amples were lyophilized, re-suspended in 50% MeOH, filtered via 0.22-µm Spin-X cartridges. The resolution was on reversed phase C_{18} column with 0-50% ACN in 0.4% CH_3COOH. Bi et al. [23] used a sensitive and simple HPLC amiloride in urine of human. The limit and recovery of detection were 0.12 µgmL^{-1} and 80.4-85.5% at different quantities. The entire urine excretion was after 24 h after oral take up of 5.0 or 15.0 mg. The drug was analyzed in urine of human up to approximately 44 h after medicine take up..

3.2.2 Metoprolol

Metoprolol is marketed as lopressor among others, a medicine of selective β_1 receptor blocker type [24]. Metoprolol was first made available in 1969 and is on the World Health Organization's List of Essential Medicines, the mainly significant medications required in a basic health system. In 2013, metoprolol was the 19th-most prescribed medication in the United States. It is utilized for high blood pressure, pain chest due to poor blood flow to the heart, and a number of conditions

involving an abnormally fast heart rate. It is also used to prevent further heart problems after myocardial infarction and to prevent headaches in those with migraines. It is sold in formulations that can be taken by mouth or given intravenously. The common side effects include trouble sleeping, feeling tired, feeling faint, and abdominal discomfort. Large doses may cause serious toxicity [25]. It appears to be safe in breastfeeding. Greater care is required with use in those with liver problems or asthma. Stopping this drug should be done slowly to decrease the risk of further health problems. Therefore the analysis of metoprolol is important in various biological samples. Some papers describing analysis of metoprolol are described below.

Hemmati et al. [26] described analysis of metoprolol by tandem dispersive liquid liquid micro-extraction and HPLC in plasma of human and pharmaceutical waste water. The best values were establish 11.0 % (w/v) of the salt quantity, an original pH value of 12.0, 103 μL of organic extracting phase, and 45 μL of water extracting phase with pH value of 2.0, consequenced in realistic revival fractions with a rational appeal. Under most favorable experimentation circumstances, good quality linear variety and small limits of detection were obtainable. Filipe et al. [27] described metoprolol analysis wastewater treatment plants using HPLC-UV-ESI-MSn. This learning examined the degraded of metoprolol in simulated solar radiation and in the company of fulvic acids extracted from a river. Throughout the photo-degradation examination the creation of new molecules was seen. Rao et al. [28] described a simple, specific and sensitive LC-MS/MS technique for the estimation of metoprolol in plasma of rat. The plasma samples were ready by

protein precipitation, then the severance of the analytes was executed on C_{18} column with 1.0 $mLmin^{-1}$ flow. The technique was completely authenticated for terms accuracy, linearity, selectivity, stability, matrix effect, precision, and recovery in 3.42-7000 $ngmL^{-1}$ concentration range. The technique was productively practical to herb drug interface learning of metoprolol.

Bae et al. [29] reported a quick and easy LC/MS/MS technique for the analysis of metoprolol and its two metabolites *i.e.* α-hydroxy- and *O*-desmethyl metoprolol in human plasma. Metoprolol, two metabolites, and internal standard chlorpropamide were taken out from plasma (50 μL) by $CH_3COOCH_2CH_3$. LC was carried out a Luna CN stationary phase with an isocratic eluent having distilled water and MeOH having 0.1% HCOOH (60:40, v/v) with 0.3 $mLmin^{-1}$ flow rate. The linear ranges of application of metoprolol, α-hydroxymetoprolol, and *O*-desmethylmetoprolol were 2-1000, 2-500, and 2-500 $ngmL^{-1}$, respectively, with a low limit of detection of 2 $ngmL^{-1}$ for all analytes. Finally, the technique was productively used to review the persuade otypes on the kinetics of metoprolol following mouth admin of 100 mg to healthy Korean volunteers. Xu et al. [30] reported a specific, sensitive, and simple HPLC method for the estimation of metoprolol and its two metabolites in urine and plasma of human. The resolution of metoprolol, α-hydroxymetoprolol, O-desmethylmetoprolol and esmolol (internal standard) was attained on an Agilent XDB-C_{18} stationary phase with fluorescence detection at 216 nm (exn) and 312 nm (emn). The eluent was ACN-H_2O-0.1%TFA. The examination was executed in fewer than sixteen min with 0.8 $mLmin^{-1}$ flow rate. The LOQ were 2.5 and 5.0 $ngmL^{-1}$ for urine and plasma, correspondingly.

Good quality accuracy and precision for metoprolol and its two metabolites were achieved. The removal revival was established to be extra than 86.91% both in urine and plasma. At the same time, the technique was productively used to 9 vigorous volunteers agreed to a tablet of metoprolol (100 mg). Thakker et al. [31] accounted a easy, quick, precise and isocratic RP-HPLC technique for the estimation of metoprolol tartrate in bulk medicine and pharmaceutical dosage form. The quantification was carried out using YMC-Pack CN (250 × 4.6 mm, 5.0 μm) column and the eluent comprises of 0.05% F_3COOH and ACN (70:30, v/v). The flow rate was 1.0 $mLmin^{-1}$. The eluent was monitored at 220 nm. The technique was authenticated in terms of accuracy, specificity, linearity, and exactness, limit of detection and limit of quantitation.

Kallem et al. [32] described a extremely responsive and specific LC-ESI-MS/MS technique for quantification of metoprolol in plasma of rat (50 μL) utilizing internal standard of phenacetin according to Food and Drug Administration regulations. Liquid liquid taking out technique was utilized to take out the analytes and internal standard in plasma of plasma. The chromatographic separation was attained with an eluent having 0.2% HCOOC in H_2O-ACN (25:75, v/v) with a time program flow gradient on a C_{18} stationary phase. A linear retort purpose was recognized for the range of quantities 0.59-1148 and 0.53-991 $ngmL^{-1}$ in plasma of rat. The validated technique was adopted to a kinetic study in rats. Zoerner et al. [33] used UPLC-MS/MS system for measuring metoprolol. Sample handling of 200 μL plasma of human, having a single liquid-liquid extraction stride by $CH_3COOCH_2CH_3$ was carried out. Chromatographic resolution was done on a

50 mm long BEH C_{18} stationary phase with gradient elution via a mixture of H_2O and MeOH having 2 mM NH_4COOCH_3 above 4.5 min. The method was authenticated according to the European Medicines Agency (EMA) regulations on bio-analytical technique validated above the range of 1.0 to 500.0 $ngmL^{-1}$. Linear rejoinders with correlation constants >0.99 above that assortment were obtained. The LOQ value was 1.0 $ngmL^{-1}$. The interior typical normalized matrix issue was close up to 1.0 for low and high analyte qauntities. Liu et al. [34] reported a specific UPLC-MS technique for the estimation of metoprolol in plasma of rat. The chromatographic resolution was obtained on HILIC C_{18} stationary phase. The eluent was made of ACN and H_2O having 0.1% HCOOH in the ratio of 15:85, (v/v). The samples of plasma were extracting using CH_2Cl_2-BuOH in the ratio of 10:1 (v/v). The calibration plots in plasma were linear in the quantities series of 2.5 to 250 $ngmL^{-1}$ metoprolol. The analysis was having correlation coefficient r2 as 0.9998. The technique was effectively used to study kinetic of the reported drug.

Rawool et al. [35] reported RP-HPLC of metoprolol tartrate present in a tablet formulation. It was a easy, fast, truthful and accurate HPLC technique. It was performed using phosphate buffer along with methanol as mobile phase, in the proportion of 60:40. The separation as done on a C_{18} column and it is estimated at 226 nm with 1.0 $mLmin^{-1}$ a flow. The specificity for interference of any peak with main peak of interest was checked. The technique was established to be accurate and precise for estimation of the two drugs simultaneously. Phale et al. [36] utilized an HPLC technique for estimation of metoprolol tartrate in the company of products produced in a strain dilapidation study. The victorious resolution of the

drug from its degradation products produced under strain situation was obtained on a C_{18} stationary phase utilizing Na_2PO_4 buffer-CAN (70:30) eluent. The flow was 1.0 $mLmin^{-1}$ with 274 nm as detection. The technique was authenticated for accuracy, precision, LOQ, and LOD and linearity, range. As technique efficiently resolved the drugs from their degradation products, it was utilized as a constancy showing technique. Yilmaz et al. [37] described a specific, sensitive and simple HPLC technique for the estimation of metoprolol in urine and plasma of human. The resolution of metoprolol and atenolol (internal standard) was obtained on an Ace C_{18} stationary phase with 276 nm (extn) and 296 nm (emsn) fluorescence detection. The eluent had MeHO-H_2O (50:50, v/v) having 0.1% TFA. The eluent was in 1.0 $mLmin^{-1}$ flow rate. The LOD and LOQ were 1.0, 1.5, 3.0 and 5.0 $ngmL^{-1}$ for plasma and urine, correspondingly. Also, the technique was productively utilized to 3 patients with high blood pressure those had been given metoprolol (100 mg). Baranowska and Wilczek [38] used HPLC technique for the estimation of metoprolol in urine of human. Analysis was carried out on a reversed-phase LiChroCART Purospher C_{18} stationary phase.

Garg et al. [39] described simple, accurate, economical, and reproducible RP-HPLC methods for assessment of metoprolol tartrate. The developed HPLC technique used a reversed-phase C_{18} column and methanol-water (95 + 5) mobile phase at an ambient temperature of 27 ± 2°C and UV detection at 225 nm; the run time was 10 min, and quantification was based on peak area. The proposed methods were effectively utilized for the assessment of metoprolol tartrate in bulk powder and dosage form. Dongre et al. [40] reported a precise, specific, simple, and exact

reverse phase HPLC technique for the assessment of metoprolol tartrate in tablet. The chromatographic resolution was obtained on Hypersil BDS cyano (250 mm x 4.6 mm, 5 micro m) stationary phase with PDA detection. The eluent having buffer (aqueous (CH_3CH_2)N pH 3) and ACN in the ratio of 85:15 (v/v) at 1.0 $mLmin^{-1}$ flow was adopted. The technique was authenticated according as per ICH regulations with respect to accuracy, linearity, precision, robustness and specificity.

3.2.3 Hydrochlorothiazide

Hydrochlorothiazide is a diuretic medication often used to treat high blood pressure and swelling due to fluid build-up. Other uses include diabetes in sipidus, renal tubular acidosis, and to decrease the risk of kidney stones in those with high calcium level in the urine. For high blood pressure, it is often recommended as a first line treatment. The potential side effects include poor kidney function, electrolyte imbalances especially low blood potassium and less commonly low blood sodium, gout, high blood sugar, and feeling faint initially upon standing up. While allergies are reported to occur more often in those with allergies to sulfa drugs this association is not well supported. It may be used during pregnancy but is not a first line medication in this group. It is in the thiazide medication class and acts by decreasing the kidneys' skill to hold water. This initially decreases blood volume, decreasing blood go back to the heart and, hence, cardiac productivity [41]. Long term, however, it is supposed to low tangential vascular fight.

Zeid et al. [42] reported used HPLC for analysis of hydrochlorothiazide immediate release in tablets. The dissolution profiles were conducted at pH values 1.2, 4.5 and 6.8. The difference factor (f1) and similarity factor (f2) were

calculated. The examined item for consumptions was effectively fulfilled with pharmacopeial needs. Abd Ęl-Hay et al. [43] used a novel, simple and robust HPLC method for assessment of hydrochlorothiazide in their bulk powders and dose forms. Chromatographic separation was carried out in less than two minutes. The resolution was performed on a RP C_{18} stationary phase with an isocratic elution system consisting of 0.03 $molL^{-1}$ *o*-H_3PO_4 (pH 2.3) and ACN as the mobile phase in the ratio of 50:50, at 2.0 $mLmin^{-1}$ flow rate at room temperature. Detection was performed at 220 nm. Validation was performed concerning system suitability, limits of detection and quantitation, accuracy, precision, linearity and robustness. Calibration plots were rectilinear over the choice of 0.195-100 μgmL^{-1} for all the drugs studied. The technique was applied to estimation of the studied analyte in pharmaceutical dosage forms. Karvelis et al. [44] reported a novel, fast, and sensitive stability showing HPLC technique for the estimation of hydrochlorothiazide in a combined dose. Effective chromatographic resolution was obtained using a phenyl analytical column with isocratic elution using the eluent 0.030 M ammonium acetate-ACN (60 + 40, v/v) at a flow rate of 0.40 $mLmin^{-1}$. The process was linear on the amount ranges of 0.125-0.375 μgmL^{-1} for hydrochlorothiazide. The intraday and inter-day RSD values were fewer than 6.1%. The planned process confirmed to be stabile showing by resolution of the drug from its forced degraded by-products. The technique was utilized productively to the QC and content uniformity tests in combined commercial tablets.

Alanazi et al. [45] reported a easy, responsive and accurate HPLC method with high throughput for the assessment of hydrochlorothiazide in combined

pharmaceutical dosage forms. The planned technique employed, for the first time, a monolithic column in the analysis. Optimal chromatographic separation of the analytes was obtained on RP-18e stationary phase utilizing an eluent of phosphate buffer (pH 4)/ACN (50:50, v/v) flowed isocratically at 1.0 $mLmin^{-1}$. In the most favorable situations, linear connection with a high-quality association constant ($R \geq 0.9997$) was established between area of the peak and the equivalent amount. The limits of detection were 0.03 $ngmL^{-1}$. The correctness of the proposed technique was > 97 %. The planned process had elevated throughput as the examination concerned an easy process and an awfully petite run-time of < 3 min. The results demonstrated that the technique was appropriate in the excellence control of combined tablets containing this drug. Koyuturk et al. [46] reported the analysis of hydrochlorothiazide by a narrative dilute and shoot HPLC. The process was done utilizing a second generation C_{18} monolithic silica stationary phase. Urine samples were set up into the machine readily, with merely filtration and succeeding dilution. The authentication learning was carried out as per the official advice and the industrial technique was effectively utilized to tablets and samples of urine. Hammouda et al. [47] described a fast HPLC technique for hydrochlorothiazide in pharmaceutical preparation. The resolution was done on a stationary phase with cyano-bonded accepting 210 nm detection with 1.0 $mLmin^{-1}$ flow. The authentication micro-emulsion eluent had of 0.2 M sodium dodecyl sulfate, 1% octanol, 10% n-$CH_3CH_2CH_2OH$ and 0.3% $(CH_3CH_2)_3NH_2$ in 0.02 M H_3PO_4, and pH was attuned at 3.5. The planned technique was established to be linear on the quantities ranges 1-100 with a correlation constant 0.9999 for both drugs. The

industrial technique was authenticated for accuracy, specificity, low limit of quantification and detection, precision and linearity. The mean revival of the analyte in tablet was in accord with those achieved from different tests.

Qui et al. [48] accounted a U-HPLC-MS/MS method to determine hydrochlorothiazide in plasma of human. The samples of plasma were made utilizing protein precipitation with CAN. Two analytes and the internal standard losartan were resolved on an Acquity U-HPLC BEH C_{18} stationary phase utilizing MS detector. The examination time per sample was 2.5 min. The urbanized and authenticated method was effectively utilized to a bioequivalence examination of tablet in Chinese healthy volunteers (N=20). Hafez et al. [49] reported HPLC analysis of hydrochlorothiazid. The method was performed by RP-HPLC utilizing an eluent made of 0.025 M $KHPO_4$ (pH 6.0): CAN (65:35%) with 220 nm on an ACE C_{18} column stationary phase at 1.5 $mLmin^{-1}$ flow rate. The planned technique was authenticated as per ICH regulations for correctness, exactness, linearity, robustness, limit of recognition and limit of quantitation. Jain et al. [50] described an easy, precise, correct and stability showing RP-HPLC for evaluation of hydrochlorothiazide in tablet form. The technique was industrialized utilizing an RP C_{18} base deactivated silica stationary phase with an eluent having $CH_3CH_2NH_2$ (pH 3.0) attuned with *o*-H_3PO_4 (A) and ACN (B), with a timed gradient program of T/% B: 0/30, 7/70, 8/30, 10/30 at 1.4 $mLmin^{-1}$ flow rate with 236 nm detection. The planned method was authenticated for accurateness, exactness, linearity, range, ruggedness, robustness, and strength degraded examination.

3.2.4 Carvedilol

Carvedilol is traded beneath the brand name Coreg and it is a beta blocker utilized for treating mild to harsh congestive heart failure (CHF), left ventricular dysfunction (LVD) following heart attack in people who are otherwise stable, and for treating high blood pressure. Beta blockers block the beta receptors on heart muscle and other cells, making them more relaxed and less responsive to stress hormones. Carvedilol also blocks alpha receptors, which are found on blood vessels, and relaxes the blood vessels, dilating them, which lowers blood pressure and vascular resistance. It is a nonselective beta blocker/alpha-1 blocker and belongs to the third generation of beta blockers.

Ballesteros-Esteban et al. [51] accounted LC-MS/MS for analysis of carvedilol in urine samples. A new method based on the combination of dispersive micro-solid phase extraction and LC-MS/MS has been industrialized to estimate carvedilol in urine samples. For this purpose a magnetic-polyamide composite was synthesized and used as sorbent. Working under the optimum conditions, the method provided limits of quantification and detection in the range of 0.1-0.15μgL^{1} for carvedilol and propranolol. Huang et al. [52] established a fast and responsive UPLC-MS-MS technique for the estimation of carvedilol and its metabolite 4-hydroxyphenyl in plasma of rat. The analyses were done on an Acquity UPLC BEH C_{18} stationary phase utilizing gradient elution with an eluent of ACN and 0.1% HCOOH in H_2O at a 0.4 $mLmin^{-1}$ flow. The technique was effectively used to a kinetic reaction of carvedilol after mouth admin of carvedilol in rats. The outcomes showed that the co-administration of carvedilol producted in a significant drug

reaction in rats. Yilmaz and Arslan [53] reported an easy, quick and responsive HPLC technique to enumerate carvedilol in plasma of human utilizing fluorescence detection. The technique had a solitary step liquid liquid extraction with $CH_3CH_2OCH_2CH_3$ and $CH_3CH_2COOCH_3$ (3:1, v/v). HPLC separation was done out by RP-HPLC with an eluent made of 20 mM phosphate buffer (pH 7)-ACN (65 : 35), flowed at a flow 1.00 $mLmin^{-1}$ with detection at 240 nm (exn) and 330 nm (emn). The limits of quantification and detection of carvedilol were 10 and 3.0 $ngmL^{-1}$, correspondingly. Besides, the technique was effectively utilized to 3 patients with high blood pressure that had given 25 mg tablet of carvedilol.

Li et al. [54] reported a rapid UPLC-MS/MS for the estimation of carvedilol, and its 3 metabolites viz. 4'-hydroxyphenyl-carvedilol, 5'-hydroxyphenyl-carvedilol, o-desmethyl-carvedilol. The efficient UPLC-MS/MS resolution of the evaluated molecules was utilized using Acquity BEH C_{18} stationary phase with a gradient mobile eluent. The examination was done in fewer than 6 min at a 0.4 $mLmin^{-1}$ flow. The test was authenticated with amount choices of 0.500-100 $ngmL^{-1}$ for carvedilol and 0.0500-10.0 $ngmL^{-1}$ for its three metabolites. Intra- and inter-assay precision magnitudes for duplicate excellence manage samples were inside 11.4% for all molecules throughout the technique authentication. Mean excellence management accurateness values were inside ±11.5% of titular magnitudes for all molecules. Technique revivals were elevated (>91%) and inner set regularized matrix properties were smallest. The authenticated technique was effectively utilized to the quantification of carvedilol and its pharmacologically vigorous metabolites in rat kinetic study. Patel et al. [55]

described an UPLC-MS-MS technique for the estimation of carvedilol and its pharmacologically vigorous metabolite in plasma of human. The sections were handeled by solid phase extraction utilizing 100.00 μL plasma of human. The resolution was done utilizing C_{18} stationary phase with using ACN-4.0 mM NH_4COOCH_3, pH 3.0 adjusted with 0.1% HCOOH (78 : 22) as the eluent. The manifold reaction checking evolution for both the analytes and IS where observe in the +(ve) electrospray ionization manner. The technique was effectively utilized to hold up a bioequivalence examination of 12.5 mg carvedilol tablets in 34 healthy patients. Soltani et al. [56] described a easy, exact and susceptible HPLC technique for the estimation of carvedilol in plasma and urine of human samples. The analyte was taken out by a dispersive liquid liquid micro extraction technique. An eluent of 15 mM Na_2HPO_4 buffer (pH 4.0)/ACN/2-PrOH (70 / 27.5 / 2.5, v/v) was utilized to resolve the drugs utilizing ODS stationary phase with 222 nm detection.

Rathod et al. [57] reported an easy, exact and HPLC technique for estimation of carvedilol in plasma of human. The technique was produced on Lichrosphere R CN stationary phase utilizing eluent of ACN/20 mM NH_4COOCH_3 buffer with 0.1% $(CH_3CH_2)NH_2$ (pH 4.5) (40 / 60, v/v). The peaks were noticed utilizing fluorescence detector (282 nm extn) and (340 nm emsn). This technique was utilized to estimate the carvedilol in plasma of human collected from patients that had admin orally 12.5 mg carvedilol tablet. Źarghi et al. [58] described an easy, quick and responsive HPLC technique with detecting by fluorescence utilizing monolithic column in plasma of human. The resolution done on a Chromolith stationary phase with an isocratic eluent having 0.01 M Na_2HPO_4 buffer−ACN

(40:60, v/v) fixed to pH 3.5. The sample handling engaged protein precipitation technique and complete revival. The technique made the estimation of carvedilol for therapeutic drug estimation with a least amount quantification limit (LOQ) of 1.0 $ngmL^{-1}$. The calibration plot was linear on amount choice 1−80 $ngmL^{-1}$. The constants difference for inter day and intra day techniques were established to be fewer than 8.0%. Machida et al. [59] reported a responsive technique for calculating levels of plasma of carvedilol in human utilizing HPLC (electrochemical detection).

3.2.5 Amlodipine

Amlodipine is traded in the brand name of Norvasc and is a medicine used to treat high blood pressure and coronary artery disease. Amlodipine was first patented in 1986 with commercial sale beginning in 1990. It is on the WHO list of essential medicines, the most effective and safe medicines needed in a health system. While calcium channel blockers are not typically recommended in heart failure, amlodipine may be used if other medications are not sufficient for high blood pressure or heart related chest pain. The common side effects include swelling, feeling tired, abdominal pain, and nausea. The serious side effects may include low blood pressure or a heart attack. It is unclear if use is safe during pregnancy or breastfeeding. The doses should be decreased in people with liver problems and in elderly individuals. Amlodipine is a protracted working calcium channel blocker of the dihydropyridine type. It works partly by increasing the size of arteries.

El-Bagary et al. [60] mentioned a simple, correct, and particular LC method with a reversed stationary phase for the dedication of amlodipine. Chromatographic extraction was carried out on a BDS Hypersil® C_{18} Stationary Phase. The cell segment, which includes 0.05 M KH_2PO_4 buffer (pH 2.6)-MeOH (50:50, v/v), became pumped through the stationary phase whose temperature turned into maintained at 50°C at a glide rate of 0.6 $mLmin^{-1}$ by using isocratic elution, and UV detection at 215 nm was achieved. The projected chromatographic process became statistically as compared to that of reference methods using one-way analysis of variance. Talele and Porwal [61] defined an easy, economical and strong analytical HPLC-UV process for chromatographic elution of amlodipine in organic biological for the first time. The bioanalytical approach became advanced in rat plasma with the aid of using Thermo beta-fundamental C_{18} stationary phase and eluent was composed of dibasic buffer of phosphate (pH < 4) : acetonitrile in the ratio of 55:45 at a glide rate of 1.0 $mLmin^{-1}$ with UV detection checked at 240 nm. The imply recovery ® % become extra than 92.8% the use of protein precipitation method. The purity of samples for 6 repeat measurements at lesser restrict of quantitation level became within limit. The approach turned into established and turned into effectively implemented to the nonclinical pharmacokinetic take a look at of combination tablets having amlodipine in six Sprague Dawley rats. Vojta et al. [62] found a novel fast stability showing UPLC approach for analysis and identification of amlodipine besilate impurities, mixed pill dosage. Zorbax Eclipse C8 RRHD stationary phase become used as a separation stationary phase and the components were separated inside 11 min via

an automatic ramp combination of 0.01M phosphate buffer (pH < 3) and ACN. The approach turned into efficiently demonstrated in accordance to the worldwide. The approach changed into found to be particular, linear, correct, touchy, particular, sturdy and stability-indicating and can be castoff as an ordinary cleanliness test approach for amlodipine besilate in pharmaceutical mixtures.

Manzoor et al. [63] used HPLC-UV for measurable estimation of amlodipine besilate in a capsule origination affording to ICH strategies. Chromatographic separation of the medicine was carried out by a Spherisorb ODS2 reverse stationary phase by means of a eluent, which be made up of 70: 30 (v/v) ACN mixture and TEA phosphate buffer (pH 3; 0.015 M) with final pH accustomed to 2.5 by means of dilute ortho-H3PO4, at 1.0 $mLmin^{-1}$ flow rate. The analytes were noticed at UV wavelength of 237 nm. This simple and convenient process could be simply applied for the description and quantitation of the medicine in a solo formulation for mixture treatment of cardiovascular diseases. Mannemala and Nagarajan [64] developed an easy, single and selective HPLC-PDA process for the approximation of amlodipine in plasma of human. The composites were analysed by HPLC by means of PDA detection with an eluent comprising ACN and buffer of phosphate (pH 4.2 and 25 mm; 60:40 v/v) with a flow rate of 0.8 $mLmin^{-1}$ on a Hibar C_{18} stationary phase. The dissimilar example pretreatment processes were estimated but precipitation of protein was originated to be suitable. The LOQ was 30 $ngmL^{-1}$. The dissimilar ingredients of proteins in plasma did not restrict with the complete recovery. Moussa et al. [65] determined amlodipine besilate in tablets using HPLC-fluorescence recognition processs. In the HPLC process,

separation was completed within 8 minutes on a C_{18} stationary phase using the eluent ACN:phosphate buffer (0.015 M, pH 3) (45:55, v/v). Fluorescence recognition was carried out using excitation wavelengths 361 and 274 nm. The excellent linearity was observed. The careful validation proved advantages of the new process: high sensitivity, accuracy, selectivity and suitability for quality control laboratories.

Qi et al. [66] determined amlodipine levels in plasma of human and urine of human by developing a delicate and rapid UPLC-MS/MS process. The parting was carried out on an Acquity UPLC BEH C_{18} stationary and mass spectrometric analysis was achieved. The assay approach has been absolutely verified in terms of discrimination, linearity, decrease LOQ, exactness, accuracy, balance, medium effect & recuperation. The linearity of this approach turned into observed to be inside the attention range of 0.1-50 $ngmL^{-1}$ and 2-1000 $ngmL^{-1}$ for amlodipine in plasma and urine of human. Özdemir and Akyüz [67] explained a brand novel, easy, fast and unique reversed section HPLC approach for the instantaneous identification of amlodipine besilate. The HPLC analysis become achieved on RP-18 stationary phase by using TEA-ortho-H_3PO_4 buffer (pH 3.0, 50 mM), ACN and MeOH (50 : 40 : 10, v/v) as an eluent at a glide rate of 1.0 $mLmin^{-1}$. The process turned into verified for specificity, exactness, accuracy, linearity and robustness. For the HPLC process, other things such as linearity degree of the calibration curves, the percent healing magnitudes of amlodipine and the limit of recognition (LOD) and limit of quantification (LOQ) have also been decided. The projected technique become efficiently implemented to amlodipine besilate in medical dosage

combinations without any interfering from the excipients. The approach turned into found to be exact, correct, reproducible & strong. The outcomes decided with those acquired using the advanced reference technique. Zhou et al. [68] noticed a touchy, easy & fast HPLC-ESI-MS/MS approach for the analysis of amlodipine in plasma of human. The analyte turned into extracted from plasma of human thru liquid-liquid extraction approach. A methyl tert-butyl ether and ethyl acetate (50:50, v/v) combination was used because the extractant. The chromatographic estimation turned into reached on a Capcellpak CR 1:4 stationary phase within 6.0 min with ACN & CH_3COONH_4 buffer (20mM) containing zero.3% formic acid (50:50, v/v) as eluent. The technique turned into established and was successfully carried out to the bioequivalence take a look at of mixture capsules.

3.2.6 Frusemide

Frusemide is vended under the product name Lasix between other and is a tablet used to treat fluid build-up due to heart letdown, liver mutilating, or kidney illness. Frusemide was discovered in 1962 and WHO recognized it in its list of essential medicines, the most effective and safe medicines needed in a health system. It may also be castoff for the dealing of elevated blood pressure. The amount of medication required depends on the person in question. It can be taken intravenously or by mouth. When taken by mouth it typically begins working within an hour while intravenously it typically begins working within five minutes. The common side effects include low blood pressure with standing, ringing in the ears, and sensitivity to the sun. Potentially serious side effects include electrolyte abnormalities, low blood pressure, and hearing loss. Blood tests are recommended

regularly for those on treatment. Frusemide is a type of loop diuretic that works by decreasing the reabsorption of sodium by the kidneys [69].

Zhang et al. [70] developed high performance liquid chromatography for direct online sample injection and recognition of frusemide in plasma and urine in rats. The stationary phase of restricted access media was used as the pre-treatment stationary phase and a C_{18} stationary phase as the analytical stationary phase. Water-MeOH (95:5, v/v) with a volume percentage of formic acid of 0.1% was used as eluent of the pre- treatment stationary phase. The eluent of the analytical stationary phase was MeOH-water (65:35, v/v) for plasma and MeOH-water (55:45, V/V) for urine samples, all containing a volume percentage of formic acid of 0.1% with 1 ml min^{-1} flow rate. The λ_{max} was 274 nm and the stationary phase temp. was preserved at 25 ºC. The average recoveries of frusemide at 3 spiked levels ranged from 101.82% to 113.36% for plasma and from 98.75% to 112.27% for urine samples. The recognition showed good intra- and inter-day assay accuracies and accuracies with the comparative standard deviations all below 5%. The pharmacokinetic parameters AUC (0→24) was 6.265 g/(ml/h) with a t(1/2) of 2.447 h and a C(Max) of 1.414 gmL^{-1}. The mean cumulative excretory rate of frusemide in the urine of rats over 24 h was 32.50%−39.08%. The recognition of frusemide in plasma and urine samples using restricted access media-HPLC is simple and efficient and allows direct online injection of the samples. Youm I, Youan BB. [71] developed a simple, sensitive, and specific process for frusemide analysis by reverse-phase-HPLC was developed using a Spherisorb C_{18} ODS 2 stationary phase. A chromatographic analysis was done by using ACN and 10 mM

K3PO4 buffer solution: 70 : 30 (v/v) as an eluent at pH 3.85, at a 1.0 $mLmin^{-1}$ flow rate. The UV-recognition process was carried out at 233 nm at room temperature. Validation constraints including limit of recognition (LOD), limit of quantitation (LOQ), linearity range, precision, accuracy, robustness, and specificity were investigated. Results indicated that the calibration curve was linear (r (2) = 0.9997) in the range of 5.2 to 25,000 $ngmL^{-1}$, with ε value equal to 3.74×10^4 L/M/cm. The two parameters such as LOD and LOQ were found to be 5.2 and 15.8 $ngmL^{-1}$, correspondingly. The established process was found to be exact (RSD less than 2%), precise, and specific with an intraday and interday RSD range of 1.233−1.509 and 1.615 to 1.963%. This process may be routinely used for the measurable analysis of frusemide from nanocariers, USP tablets and release media related to hearing research. Ram et al. [72] accounted RP-HPLC process for the analysis of frusemide in durable pharmaceutical dosage paperwork. Separation involving isocratic RP-HPLC, became finished on an SGE SS Wakosil ÌÌ 5C8 RS 5−μm stationary phase using an eluent of ACN-CH_3COONH_4 buffer (50:50, v/v) at a 1.0 $mLmin^{-1}$ flow rate. The recognition become finished at 254 nm the usage of a photodiode array detector. The process changed into tested for precision, accuracy, specificity, linearity, robustness and answer balance. The technique turned into observed to be linear within the medicine attention range of 40−160 μgmL^{-1} with association coefficients of 0.9953 for frusemide. The accuracy (relative well known deviation; RSD) among a six-sample preparation was zero.87%. Repeatability and intermediate precision (RSD) amongst a six-sample preparation was 0.20%.

Patil et al. [73] used subtle liquid chromatographic process for the instantaneous analysis of frusemide. Chromatographic resolution was carried out by a reversed phase technique on a C_{18} stationary phase, using the eluent of water pH 3.0 and 50:50 mixtures of MeOH and ACN (58 : 42), at a flow-rate of 1.0 $mLmin^{-1}$ and a stationary temperature of 40°C. The proposed process was gauged for validation limits including linearity, choice, correctness, exactitude, limit of quantification (LOQ), limit of recognition (LOD), and specificity. The process was positively utilized for analysis of standard compounds in Caco-2 permeability experiments. Baranowska et al. [74] developed an UHPLC-UV process for the instantaneous dedication of frusemide in plasma of human and urine of human. The mobile section composed as ACN and 0.1% formic acid became used. The determined substances were eluted from a Hypersil GOLD C_{18} stationary phase in 3.3 min. Excellent linear relationships were detected (R^2) higher than 0.994. The limit of recognition (LOD) values numerous from zero.01 to 0.07 μgmL^{-1}. Galaon et al. [75] explained an easy, excessive-throughput, exceptionally discriminating and subtle HPLC-FLD approach for frusemide analysis and isolation in plasma of human sample. Separation of analyte and internal standard < 5.3 min was obtained (injection to injection) on a Chromolith performance RP-18e stationary phase, the usage of an aqueous component containing 0.015 mol L^{-1} sodium heptane-sulfonate and 0.2% TEA added to pH = 2.5 with H_3PO_4. The concerto of the mobile segment become: CAN - MeOH - aqueous component = 70 : 15 : 15 (v/v) and the flow rate turned into set up to 3.0 $mLmin^{-1}$. The overall technique primes to quantitation limits of about 27 $ngmL^{-1}$ for frusemide the use of an injection extent of 250 µL.

Wenk et al. [76] described a quick and dependable HPLC assay for the willpower of the loop diuretic frusemide in plasma and urine, using a Chromolith RP 18e monolithic silica rod HPLC stationary phase. Plasma and urine samples were estranged with a gradient along with solvent A (20% ACN) and solvent B (80% ACN), each in 0.25% acetic acid after liquid liquid extraction with CH_3OCH_3. The retention times for the inner fashionable (naproxen) and for frusemide have been 2.1 and 3.7 min, correspondingly, and 8 min was overall run time. Linear calibration curves 7.8-1000 $ngmL^{-1}$ was obsessived, and inside-assay and between-assay coefficients of version have been <6.5% and <10%, correspondingly. Using monolithic silica rod chromatography, the planned examine for frusemide in plasma and urine is sensitive, reliable, fast, and, thus, well suitable for pharmacokinetic studies.

Valizadeh [77] described a simple HPLC process. The eluent was ACN-water-TEA-glacial acetic acid (41.5 + 57.4 + 0.1 + 0.9, & adjusted to pH 5.6) at 1.0 $mLmin^{-1}$ flow rate; the run time was 9 min. The calibration graphs were linear for all 3 compounds ($r > 0.999$) throughout the attention variety of 6.25-100 μgmL^{-1}. The limits of quantitation had been 7.2, μgmL^{-1} for frusemide. El-Saharty [78] evolved a realistic, touchy, selective and green HPLC approach for the analysis of frusemide. The medicine become eluted thru a stationary phase called as Nucleosil C_{18} with an eluent composed of 0.02 M KH_2PO_4 and ACN (80:20, v/v) whose pH was adjusted to 4.5 and the effluent from the stationary changed into watched at 235 nm. The present approach enabled isocratic HPLC and easy with UV recognition of these medicines in uncooked materials and in pharmaceutical

formulations. The technique retained its accuracy and precision when making use of the usual adding process. The consequences acquired by using making use of the proposed process turned into statistically analyzed and compared with those obtained by way of the pronounced techniques. Nava-Ocampo et al. [79] described a simplified reversed section excessive HPLC approach with UV recognition at 280 nm without removal process to count frusemide in rabbit and human urine. Within and between day accuracy and precision have been always underneath 10% at all analyzed concentrations. Validation information confirmed that this technique is linear, touchy, selective, unique, correct and reproducible.

3.2.7 Telmisartan

Telmisartan, an angiotensin II receptor antagonist (angiotensin receptor blocker, ARB) is used in the organization of hypertension. It was discovered by Boehringer Ingelheim and launched in 1999 as micardis.

Mudiam et al. [80] reported the aggregate of molecularly engraved SPE and dispersive liquid liquid micro-extraction for the selective pre-concentration and resolution of telmisartan in rat urine, plasma and pharmaceutical system via HPLC. The different issues that might influence the removal performance of molecularly engraved SPE and dispersive liquid liquid micro-extraction had been optimized. In urine, the LOQ and LOD had been located to be 0.19 and 0.63 μgmL^{-1}, whilst it become observed to be zero.28 and 0.87 μgmL^{-1} in plasma, correspondingly. The % restoration of TEL in unique medium was determined to be within the variety of 81-97%. As in step with the authors, the proposed technique may additionally locate extensive submissions in scientific, qc laboratories and toxicological for the

ordinary evaluation of the medicine. Kurade et al. [81] reported a HPLC process for the telmisartan estimation in combined dosage form. A Genesis C_{18} stationary phase having dimensions of 4.6 x 250 mm and particle size of 5 mum in isocratic mode, with eluent containing a mixture of 0.01 M KH_2PO_4 buffer (whose pH was adjusted to pH 3.4) using H_3PO_4 MeOH:ACN (15:15:70, v/v) was used. The eluent was pumped at a 1.0 $mLmin^{-1}$ flow rate and the eluents were watched at 210 nm. The process was validated in terms of accuracy, linearity, specificity, and accuracy, boundary of quantitation and boundary of recognition. The process was found to be robust and can be successfully used to determine the medicine content of marketed formulations. Zhang et al. [82] described a rapid HPLC process using a monolithic stationary phase with fluorescence recognition for analysis of telmisartan in plasma of human. Preparation of sample was done by precipitation of protein with ACN and naproxen was used as IS. Fluorescence was used to detect the analytes, using an excitation wavelength of 300 nm and emission wavelength of 385 nm. Telmisartan Calibration curves were linear in the range of 1-200 $ngmL^{-1}$. The assay was high sensitive throughput, and precise, and it was positively useful to a bioequivalence study of two telmisartan formulations.

del Rosario et al. [83] gave a stationary phase-switching HPLC process for telmisartan quantification in urine of human. Samples of urine have been diluted on the elimination eluent (1:4) and 20 µL volume of this combination were directly introduced onto the HPLC system. The analyte became removed from the matrix by using an online solid phase extraction process concerning a pre-stationary section packed with 25 microm C_{18} alkyl-diol aid, and an answer 2% MeOH in 5 mM

phosphate buffer (pH < 4) at a flow rate of 0.8 $mLmin^{-1}$ for telmisartan isolation and pre-concentration. The resolution was with 5mM phosphate buffer (pH 3.8)-ACN - MeOH (65:20:15, v/v) mixture at 3.0 $mLmin^{-1}$ flow rate and detected by means of fluorescence at 259 and 399 nm as excitation and emission wavelength, correspondingly. Telmisartan separation of become finished on a Chromolith RP-18e monolithic stationary phase. The approach furnished extraction revivals from spiked urine samples more than 93%. Rane et al. [84] stated an easy, rapid, and unique process turned into developed for the measurable instantaneous analysis of telmisartan in mixed pharmaceutical dosage form. The chromatographic separation became attained on an ACE 5 C_{18} 25-cm analytical stationary phase the use of buffer-ACN (60:40, v/v) whose pH was adjusted 5.5 with CH_3COOH. The buffer utilized in eluent consists of 50 mM CH_3COONH_4 in double distilled water. The instrumental situations were 1.0 $mLmin^{-1}$ flow, temperature 30 ºC, stationary phase and 260 nm detection. Methyl paraben was utilized as an interior standard. The approach turned into verified for accuracy, linearity, accuracy, edge of quantification, limit of recognition, and robustness. The extraordinary linearity was indicated by calibration curve over the concentration variety for telmisartan. The correlation constant for telmisartan were 0.9999. The relation general deviation for 6 duplicate examinations in sets of each medicine in tablets is continually much less than 2%. The planned approach turned into determined to be fit & correct for telmisartan measurable analysis in medicinal training and it can be castoff for the quality management of components products. Shen et al. [85] gave a touchy, easy, and correct HPLC process for the assay of telmisartan in plasma of human. Using

naproxen as interior standard, the assay involved liquid-liquid extraction of the compound from acidified plasma into organic solvent and reversed-phase chromatography with fluorescence recognition. The assay was proven to be linear from 0.5 to 1000 ng ml^{-1}. In 24 wholesome volunteers, the plasma concentrations of the medicine have been decided after a unmarried oral dose of one hundred sixty mg. Torrealday et al. [86] described a HPLC approach with fluorimetric recognition for the quantitation of the telmisartan in urine, the use of a Novapak C18 stationary phase. The eluent composed of a combination ACN-phosphate buffer (45:55, v/v) (pH 6.0, 5 mM) pumped at 1/2 $mLmin^{-1}$ flow charge. Chromatographic variables have been optimized by experimental design. An easy-up step turned into used for urine samples including a solid phase removal process with C_8 cartridges and MeOH as eluent.

3.2.8 Losartan

An angiotensin II receptor antagonist medicine, Losartan is sold under the trade name Cozaar and mainly used to treat high blood pressure (hypertension). It was the first angiotensin II antagonist to be promoted. It is on the World Health Organization's list of crucial pills, the most effective and safe medicines needed in a health system. Walash et al. [87] presented a brand new, precise and touchy reversed-phase HPLC approach for the analysis of losartan potassium. top chromatographic analysis was achieved within 6.0 min on Waters, ireland and ProDIGY five 3100 A stationary section. An eluent containing a mixture of 0.02 M phosphate buffer & MeOH (65:35, v/v) at pH 3.0 become utilized. The analyses became accomplished at a go with one $mLmin^{-1}$ flow rate with fluorescence

recognition. Aspirin became used as an internal standard. The approach changed into efficiently implemented for the instantaneous evaluation of the studied pills of their laboratory-organized mixtures, unmarried medicines and co-formulated pills. Dubey et al. [88] described an HPLC technique for instantaneous analysis of losartan potassium in preparation. This approach is primarily based on a HPLC separation of the medicine on the Thermo Hypersil BDS-C_{18} stationary phase with isocratic situations and a simple eluent containing ACN-H_2O (60:40, v/v) at a go with 0.8 $mLmin^{-1}$ flow rate using UV recognition at 237 nm. This technique were applied to a advertised process without interference of excipients. The technique changed into proven for precision, robustness and healing. The statistical evaluation confirmed that the approach is repeatable and selective for the inference of losartan potassium. Rao and Srinivas [89] stated a simple, unique and precise RP-HPLC was developed for the analysis of losartan potassium in table dosage bureaucracy. A Hypersil ODS C_{18} stationary phase, in isocratic mode, with eluent ACN:MeOH:10 mM tetra butyl ammonium hydrogen sulphate in water inside the ratio of (30:30:40%, v/v) became utilized. The flow rate changed into 1.0 $mLmin^{-1}$ and effluent changed into watched at 210 nm. The linearity variety for losartan potassium turned into inside the range of 0.04-100 μgmL^{-1}. The proposed technique become additionally proven and effectively useful to the examination of losartan potassium in joint pill formulations.

Bonfilio et al. [90] described an analytical technique validation and optimization for losartan potassium analysis in tablets by using HPLC the fractional factorial and Doehlert projects. This multivariate method allowed a sizable

perfection in chromatographic presentation utilizing few experimentations, with no extra cost for stationary phases or other scheme. The HPLC approach applied K_3PO_4 buffer (pH 6.2; 58 mmol L^{-1}-ACN (65:35, v/v) as the eluent, pumped at a go with 1.0 $mLmin^{-1}$ flow rate. An octylsilane stationary phase maintained at 35 °C became used because the stationary phase segment. The limits of quantification and recognition had been 0.114 and 0.420 mgL^{-1}, correspondingly. The established approach may be utilized to count losartan potassium in medicines and to determine the stableness of this medicine. Obando et al. [91] suggested a multi-syringe flow device online coupling with a chromatographic monolithic stationary phase for analysis of losartan potassium in pills. The scheme included a multi-syringe module, 3 low pressure solenoid valves, a monolithic C_{18} stationary phase and a diode array detector. The eluent was 10 mmol L^{-1} KH_2PO_4 (pH 3.1)-ACN-MeOH (65:33:2, v/v) at 0.8 $mLmin^{-1}$ flow rate. UV recognition was performed at 226 nm. The results from validation have been superb. The approach changed into located to be appropriate to habitual evaluation of each molecules in tablets. The combination of the monolithic stationary phase with a multi syringe flow injection analysis various furnished a tremendous and cheaper device to resolve the evils of separation without use of HPLC arrangement. Ansari et al. [92] described an HPLC approach for losartan evaluation. An HPLC process became evolved for the analysis of losartan in the tablet dosage form. This process became also speedy and within your means in contrast with the extra time-eating HPLC approach often used for components screening and first-class manage. It could be used automatically by using any laboratory owning of the medicine.

Erk [93] stated a brand new easy, unique, discriminating RP-HPLC and rapid approach for resolving losartan potassium within the pharmaceutical formulations. The approach become a reversed-phase stationary phase using 0.01 N NaH_2PO_4 : MeOH : ACN (8:2:1, v/v) (pH 5.5) as an eluent with recognition at 265.0 nm. Yeung et al. [94] described an HPLC scheme composed of a (250 x 2 mm i.d. 5µ) C_{18} reversed stationary phase preceded by way of a 4 x 4 mm shield stationary phase, a UV sensor set at 254 nm, and an integrator. The eluent was a mixture of 0.01 M $(NH_4)_3PO_4$: ACN : MeOH (6:3:1, v/v) with 0.04% TEA and 0.02 % sodium azide, with pH adjusted to a 3.2. The machine changed into operated isocratically at ambient temperature at 0.3 $mLmin^{-1}$ flow rate. The HPLC assay had reproducibility, good enough, sensitivity, and specificity for clinical pharmacokinetic research. Carlucci et al. [95] defined a process for the dedication of losartan potassium in pills. The technique, based on the use of reversed-phase HPLC, become linear in the attention range 3.0-7.0 μgmL^{-1} for losartan, become easy and speedy and allowed correct and precise effects. The limit of recognition turned into 0.08 μgmL^{-1}.

3.2.9 Olmesartan

Olmesartan medoxomil, an angiotensin II receptor antagonist which has been used for the dealing of high blood pressure. It changed into developed with the aid of Sankyo in 1995, and is bought under the exchange call Benicar Olmecip. An ester promedicine, it is totally and swiftly hydrolyzed to the energetic acid form, olmesartan. Patel and Patel [96] developed an easy, speedy, and particularly selective RP-HPLC approach became developed for analysis of olmesartan

medicine materials inside the fixed dosage strength of 20 mg. Powerful chromatographic separation was finished by using a Hypersil GOLD C_{18} stationary phase with an eluent which was made of MeOH, ACN, and water (40:40:20,v/v). The eluent turned into pumped using a gradient HPLC device at 0.5 $mLmin^{-1}$ flow rate of. The reliability & analytical overall presentation of the proposed HPLC system had been statistically established with appreciate to system linearity, suitability, levels, accuracy, precision, specificity, robustness, recognition & quantification limits.

Sharma and Pancholi [97] defined an easy, sensitive and specific RP-HPLC-DAD approach for olmesartan medoxomil analysis inside the presence of its degradation products. On a C_{18} desk stationary phase, all of the degradation products and olmesartan medoxomil were resolved with the eluent composed of MeOH, ACN and water (60:15:25, v/v, pH 3.5 by means of ortho-H_3PO_4) at 260 nm the usage of a photodiode array detector. The technique was used to take a look at the medicine degradation behavior beneath forced conditions. The technique changed into implemented efficiently for olmesartan medoxomil assay inside the pill dosage form. Liu et al. [98] established a particular, touchy and speedy process primarily based on HPLC-MS-MS for olmesartan analysis in plasma of human and urine of human. Solid phase extraction became castoff to detach the molecules from biotic medium accompanied through inoculation of the extracts onto a C_{18} stationary phase with isocratic elution. The linearity, recovery, matrix effect and balance have been established in plasma of human and urine.

Sultana et al. [99] described an easy, selective, sensitive, precise, instantaneous serum samples HPLC analysis and commercial tablet formulation containing olmesartan. Proper chromatographic separation was performed the usage of a micro-Bondapak, C18 stationary phase a eluent including ACN-0.2% acetic acid aqueous solution (50:50, v/v) at a go with 1.0 $mLmin^{-1}$ flow rate. The approach used protein precipitation with ACN for the preparation of serum sample. Liu et al. [100] stated a selected, touchy and fast approach based on excessive HPLC-MS-MS for the olmesartan analysis in plasma of human and urine of human. Solid-phase extraction (SPE) turned into castoff to detach the compounds from biotic matrix observed with the aid of injection of the extracts onto a C_{18} stationary phase with isocratic elution. The process became demonstrated over the awareness variety of 0.2-1000 and 5-10,000 $ngmL^{-1}$ for olmesartan in plasma of human and urine of human, correspondingly. The process changed into useful to the pharmacokinetic examine of olmesartan medoxomil in healthy chinese male and girl subjects.

A radical seek of literature changed into performed thru Scopus, Scifinder, technology peer reviewed Journals and direct, which has been mentioned within the above paragraphs. It changed into determined that there are a few HPLC techniques for analyses of those medicines but nobody defines HPLC separation of all these molecules concurrently. Except, those strategies are time and expensive chemical consuming. Additionally, the limits of recognitions of these HPLC processs are quite high. Besides, SPE process for the instantaneously extraction of these medicines is not available so far. Keeping these facts into deliberation, the attempts have been made to validate and develop SPE and HPLC processs for the

identification and separation of all these medicines instantaneously in plasma of human. Besides, the efforts were also made to determine any new by products during combination therapy. The results of these findings are reported herein.

3.3. RESULTS AND DISCUSSION:

The results and discussion section is divided into two parts *i.e.* SPE and HPLC experiments which are discussed below.

3.3.1 Solid Phase Extraction

SPE process was used to separate the medicine mixture and any other new molecules or interferences from plasma samples. To regulate the efficiency of the reported SPE processs the % recoveries of each cardiovascular medicine were calculated. The % recoveries of amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, frusemide, telmisartan, losartan and olmesartan were resoluted by doing the blank experimentations. The calculated percentage revivals of these cardiovascular medicines from plasma are given in Table 3.1. A examination of this table point to that the magnitudes of the percentage recoveries of amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, fusemide, telmisartan, losartan and olmesartan were 60, 65, 30, 10, 30, 10, 10, 10, and 100%, respectively. The remaining amounts of these drugs (bound to plasma proteins) were 40, 35, 70, 90, 70, 90, 90, 90, and 90%, respectively. The optimization of SPE was attained by untrustworthy dissimilar new circumstances such as concentrations and pHs of phosphate buffer and the flow rates of plasma samples, phosphate buffer and eluting solvents. Instead of, other eluting solvents such as MeOH, EtOH, $CH_3COOCH_2CH_3$ and CH_2Cl_2 were also tested. Thus, by exhaustive testing, the

best eluting solvent was MeOH. It is observed that the polarity of methanol is good enough to elute these cardiovascular drugs from C_{18} cartridge. The maximum percentage revivals of cardiovascular drugs were achieved using phosphate buffer (20.0 mM, pH 7.0) separately and respectively, at 0.1 $mLmin^{-1}$ flow rate. The values of RSD, correlation constant (R) and confidence level for these drugs ranged from 1.2-1.6, 0.9994-0.9995 and 99.2-99.5, respectively (Table 3.2).

Table 3.1. The percentage recoveries of cardiovascular drugs from human plasma.

Name of drugs	% Recovery by SPE from human plasma	% of drug interactions with human plasma
Amiloride HCl	60.0	40.0
Metoprolol tartrate	65.0	35.0
Hydrochlorothiazide	30.0	70.0
Carvedilol	10.0	90.0
Amlodipine besilate	30.0	70.0
Frusemide	10.0	90.0
Telmisatan	10.0	90.0
Losartan potassium	10.0	90.0
Olmesartan	10.0	90.0

Table 3.2. Validation data of SPE method.

Drugs	%RSD	Correlation Coefficient (r)	Confidence Level (%)
Amiloride HCl	1.5	0.9995	99.5
Metoprolol tartrate	1.6	0.9995	99.5
Hydrochlorothiazide	1.2	0.9994	99.3
Carvedilol	1.3	0.9994	99.3
Amlodipine besilate	1.6	0.9995	99.5
Frusemide	1.2	0.9993	99.2
Telmisatan	1.3	0.9994	99.3
Losartan potassium	1.5	0.9995	99.5
Olmesartan	1.5	0.9995	99.5

3.3.1.1 Optimization

As usual solid phase extraction is the best tool for extraction of various molecules. The same method was applied for extraction of cardio vascular drugs in human plasma. The percentage recoveries of cardio vascular drugs from standard samples ranged between 90.0 to 95%, respectively. These values were from 10 to 65% in plasma samples. These results indicated that solid phase extraction was an optimum method for the extraction of cardio vascular drugs. The recoveries were less in plasma samples as compared to standard samples because in case of plasma samples some amount of cardio vascular drugs interacted with the protein molecules and cannot be extracted by SPE. It is also interesting to observe that in HPLC chromatograms no extra peaks were found, which shows the selectivity of the solid phase extraction method. SPE process was also optimized by pH of the

plasma, flow rate of plasma and flow rate of eluting solvent. Besides, various eluting solvents *viz*. MeOH, CH_2Cl_2, EtOH, CH_3COCH_3 were used for the optimization of the solid phase extraction method. An extensive experimentation was carried out for optimizing SPE conditions and the experimental procedure is reported in the sections below.

3.3.1.1.1 Effect of pH of Plasma

The percentage recoveries of the analytes change according to the pH of the plasma. In solid phase extraction, the purification of the analyte from the plasma occurs on the basis of adsorption on the silica gel of the C_{18} cartridge. This process of sorption is mainly ruled by the adjustment of plasma pH. The pH range from 1.0 to 10.0 was used in this removal process, to find out the variation in percentage recoveries with the change in the pH of the plasma. The Figure 3.2 shows the effect of various pH ranges on the percentage recoveries of cardio vascular drugs. It is obvious from the figure that at lower pH values the percentage recoveries are poor and augmented up to 7.0 pH. Additional augment in pH also decreased percentage recoveries in the standard solutions. The maximum percentage recoveries of the drugs at pH 7.0 were in the range of 90-95%. The reason of the various values at pH 7.0 is due to different ionic strength of thee drugs.

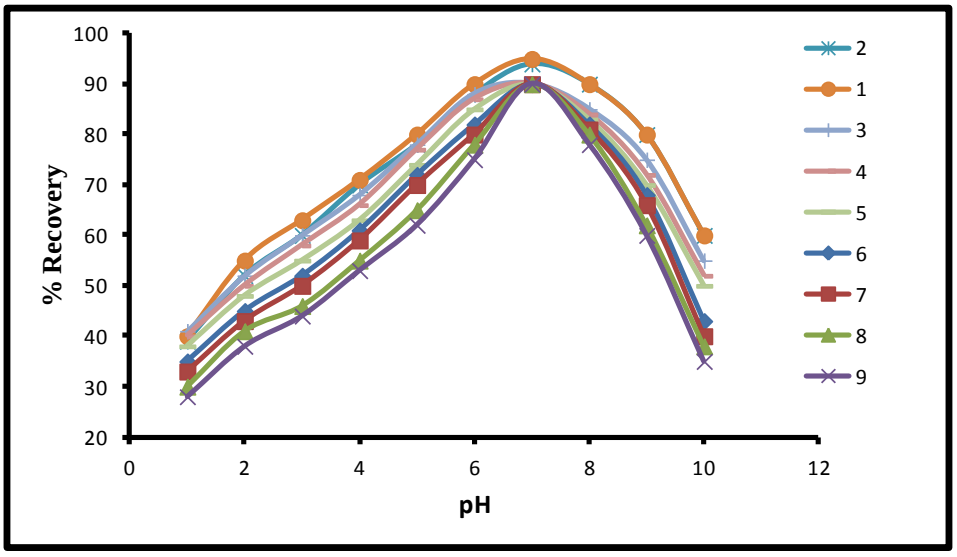


Figure 3.2: The effect of different pH of plasma on the percentage recoveries of cardiovascular drugs.

1. Amiloride, 2. Metoprolol, 3. Hydrochlorothiazide, 4. Carvedilol, 5. Amlodipine, 6. Frusemide, 7. Telmisartan, 8. Losartan and 9. Olmesartan.

3.3.1.1.2 Effect of Flow Rate of Plasma

The run rate is one of the main significant optimizing variables in solid phase extraction. Usually, elevated percentage revivals of analytes are attained at deliberate flow rate of plasma while quick run rate guides to the deprived percentage revivals of the analytes. Because of this, efforts were made towards the proper optimization of the solid phase extraction for maximum recoveries of the drugs. For this purpose a variety of flow rates like 0.025, 0.05, 0.075, 0.10, 0.125 and 0.15 $mLmin^{-1}$ were tried in the removal procedure. The outcomes of the experiments are exposed in Figure 3.3 and this figure clearly illustrates that percentage revivals of the cardio vascular drugs ranged from 99 to 64 at different flow rates. Of course, the maximum percentage revivals were at 0.025 $mLmin^{-1}$ flow rate but this flow rate was too low to use experimentally. Keeping all the facts,

into consideration, 0.10 $mLmin^{-1}$ flow rate was considered worthwhile and the maximum percentage recoveries at this flow rate were 90 to 95 percent. Therefore, this flow rate was used throughout this study.

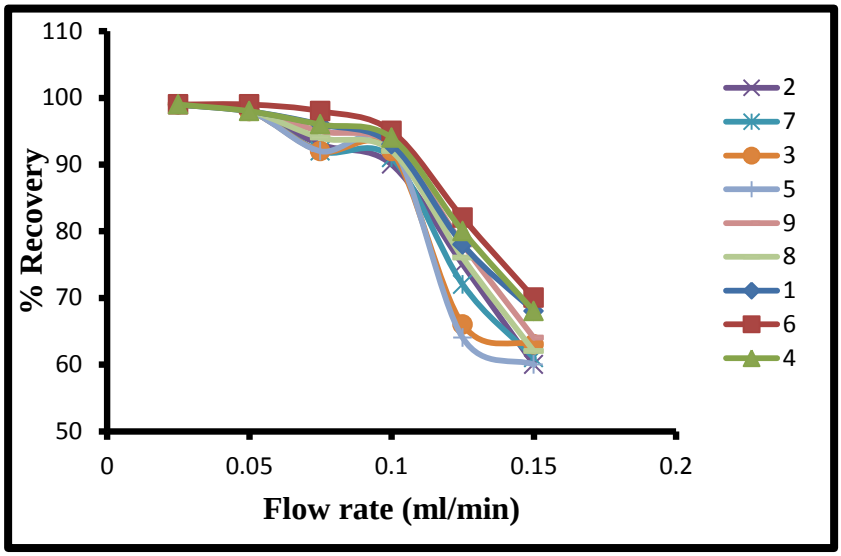


Figure 3.3: The effect of different flow rates of plasma on the percentage recoveries of cardio vascular drugs.

1. Amiloride, 2. Metoprolol, 3. Hydrochlorothiazide, 4. Carvedilol, 5. Amlodipine, 6. Frusemide, 7. Telmisartan, 8. Losartan and 9. Olmesartan.

3.3.1.1.3 Effect of Other Solvents

The selection of suitable eluting solvent also governs the optimization of the solid phase extraction. So, in order to investigate the best eluting solvent for the elution of essential amino acids through the C_{18} cartridge various solvents were tried. MeOH, CH_2Cl_2, EtOH, CH_3COCH_3 and $CH_3COOCH_2CH_3$ were used as the eluting intermediate to attain the upper limit revivals of the cardio vascular drugs from the C_{18} cartridge. The percentage recoveries of the drugs obtained by the use of these five solvents are presented in the Figure 3.4. It is very clear from this figure that the order of percentage recoveries of the analytes is MeOH > CH_2Cl_2 > EtOH > CH_3COCH_3 > $CH_3COOCH_2CH_3$. A critical evaluation of this set of experiment indicated that the greatest and least percentage revivals of the drugs were got by using MeOH and $CH_3COOCH_2CH_3$, correspondingly. Consequently, it was accomplished that methanol was the best eluting solvent for the utmost revivals of the drugs through the C_{18} cartridge. Different polarities and dielectric constant values of these solvents are responsible for their different behavior. Amid these solvents MeOH was extra talented in the procedure of desorption of the molecules during the C_{18} cartridge as it has high-quality dielectric constant assessment and polarity than that of others. Besides MeOH, CH_2Cl_2 also has substantial magnitudes of dielectric constant and polarity and, therefore, also provides high-quality percentage revivals of the drugs. But the use of methylene dichloride was avoided in the experiments because of its volatile nature, which is not safe for the experimental operator. And for the remaining solvents the magnitude of dielectric constants and polarities were not important to origin the bond dissociation between

the analytes of the reported drugs and C_{18} material of solid phase cartridge. Due to which they gave low values of percentage revivals of the reported drugs. Consequently, MeOH was proved to be the best eluting solvent and it was utilized all through the experimentations.

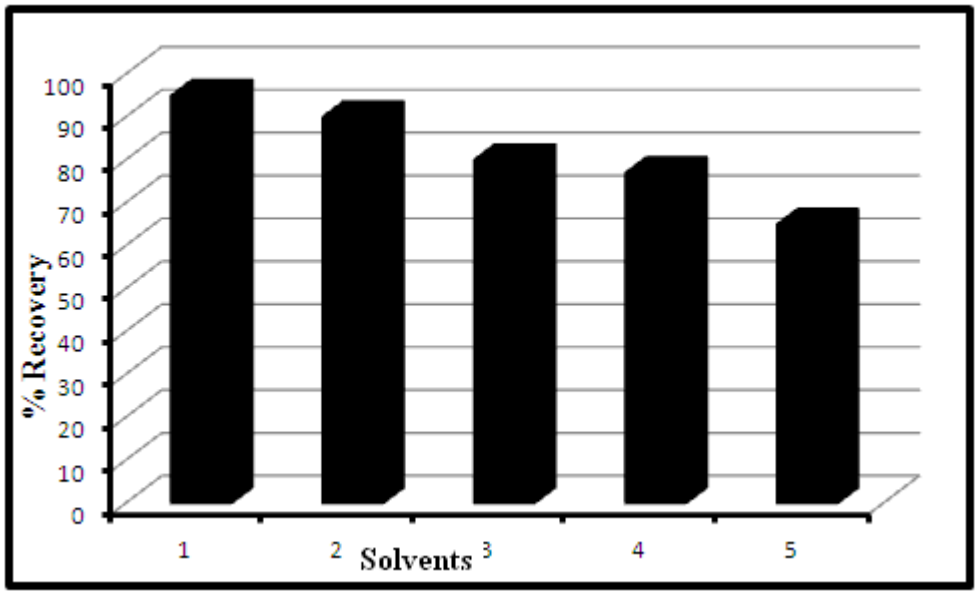


Figure 3.4: The effect of different solvents on the percentage recoveries of cardiovascular drugs.

1. Methanol, 2. Dichlorometane, 3. Ethanol, 4. Acetone, and 5. Ethyl acetate.

3.3.2 Liquid Chromatography

The chromatographic variables like retention (k), separation (α), and resolution (Rs) factors were calculated for amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, frusemide, telmisartan, losartan and olmesartan. The values of these parameters are given in Table 3.3. The values of retention, separation and resolution factors were ranged from 0.19-3.40, 1.20-3.60

and 2.43-12.37, respectively. The values of retention times, tailing factor and number of theoretical plates ranged from 2.64-11.60, 1.20-1.34 and 25883-144283, respectively. The HPLC chromatograms of these cardiovascular drugs in standard and plasma samples are given in Figure 3.5 and 3.6, respectively. It is clear from these figures that the reported drugs were base line separated. The identification of the separated drugs was determined by running and comparing the retention times of the individual amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, frusemide, telmisartan, losartan and olmesartan drugs, respectively. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported cardiovascular drugs.

Table 3.3: The values of retention times (Rt), capacity factor (k), separation factor (α) and resolution factor (Rs), tailing factor (T) and number of theoretical plates (NTP).

S.No.	Drugs	Rt	k	α	Rs	T factor	NTP/meter
1.	Amiloride HCl	2.64	..	..	..	1.33	025883
2.	Metoprolol tartrate	3.13	0.19	..	2.86	1.34	034283
3.	Hydrochlorothiazide	3.58	0.36	1.91	2.52	1.34	042686
4.	Carvedilol	6.011	1.28	3.59	12.37	1.33	084358
5.	Amlodipine besilate	6.64	1.52	1.19	2.93	1.29	096847
6.	Frusemide	7.59	1.88	1.24	4.12	1.25	106327
7.	Telmisatan	8.46	2.21	1.18	3.55	1.26	120859
8.	Losartan potassium	10.86	3.11	1.41	8.78	1.20	144150
9.	Olmesartan	11.60	3.40	1.09	2.43	1.19	144283

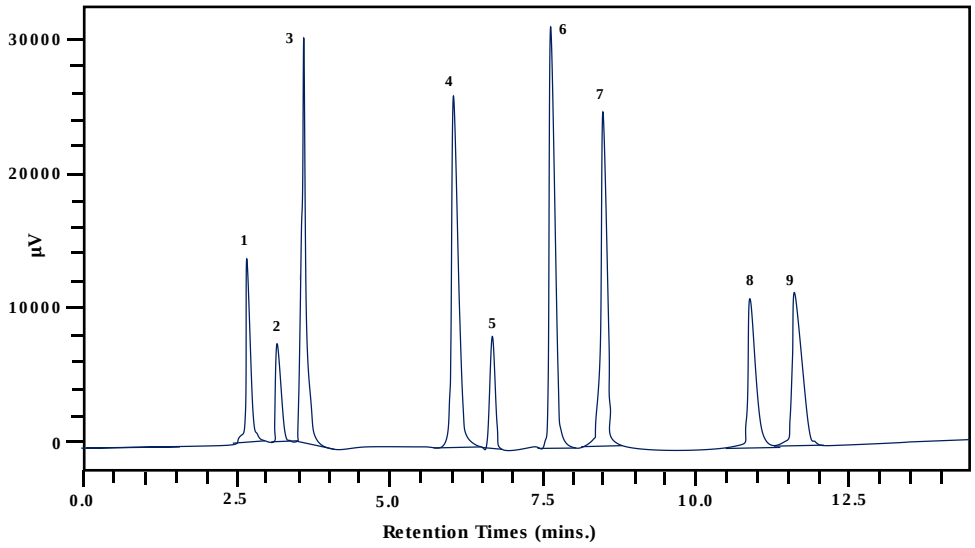


Figure 3.5: HPLC chromatogram of standard cardiovascular drugs.

1-Amiloride HCl, 2- Metoprolol tartrate, 3-Hydrochlorothiazide, 4-Carvedilol, 5-Amlodipine, 6-Frusemide, 7- Telmisartan, 8-Losartan and 9-Olmesartan.

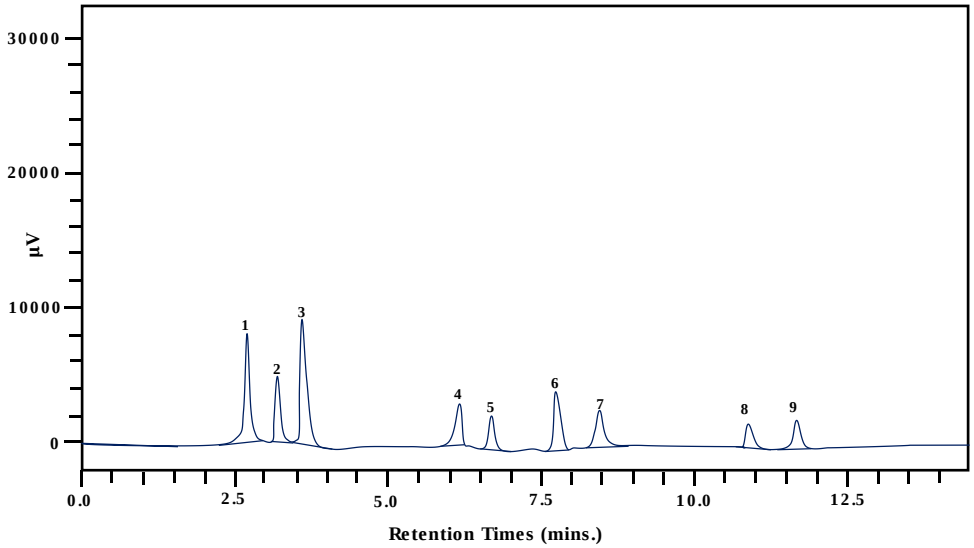


Figure 3.6: HPLC chromatogram of cardiovascular drugs in human plasma.

1-Amiloride HCl, 2- Metoprolol tartrate, 3-Hydrochlorothiazide, 4-Carvedilol, 5-Amlodipine, 6-Frusemide, 7- Telmisartan, 8-Losartan and 9-Olmesartan.

3.3.2.1 Liquid Chromatography Method Optimization

To optimize the HPLC circumstances, different combinations of eluents were tried. Besides, the modification in rate, detection and quantity inserted was also carried out. The pHs of the mobile phase were tried with triethyl amine. Additionally, optimization was also attained utilizing a few additives such as diethyl- and triethyl amines in the eluent. Owing to comprehensive experimentation, the most excellent HPLC circumstances were developed and accounted herein.

The effect of ACN on the resolution of these drugs was carried out. The amounts of ACN were varied from 10-50 mL. From the figure we can observed that the peaks were broad from 10-30 mL. Contrarily, the peaks compounded into one a new at high value of acetonitrile (40-50 mL). As a result 35 mL acetonitrile

was found suitable for the maximum separation. The flow rate of the solvent system was varied from 0.2 to 1.0 $mLmin^{-1}$. It was observed that at low flow rates (0.2, 0.3 and 0.4 mLm^{-1}), the peaks were badly determined with elevated retention times. Contrarily, at increase flow rates to 0.6-1.0 mLm^{-1} the peaks were compound into one one more. In brief, the peaks were well resolved at 0.5 $mLmin^{-1}$ flow rate, which was considered as the best one.

3.3.2.2 Validation

The results of HPLC validation parameters like linearity, LOD, LOQ, specificity, precision, accuracy, robustness and ruggedness are given herein.

3.3.2.2.1 Linearity

The linearity of calibration curves (peak area *vs.* concentration) for amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan standards were checked over the concentration ranges of 4.018-6.026 μgmL^{-1}, 4.504-6.756 μgmL^{-1} , 3.910-5.866 μgmL^{-1}, 3.936-5.894 μgmL^{-1}, 4.019-6.029 μgmL^{-1} , 3.917-5.875 μgmL^{-1} , 4.038-6.058 μgmL^{-1}, 3.936-5.904 μgmL^{-1} and 4.099-6.149 μgmL^{-1} respectively. The plotted curves were linear over these concentration ranges (n = 5) for the reported cardiovascular drugs. The peak areas of amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan were plotted *vs.* their respective concentrations. The linear regression analysis was estimated on obtained curves. The correlation coefficient (r) (n = 5) for amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan,

losartan potassium and olmesartan were found to be 0.9997, 0.9998, 0.9995, 0.9998, 0.9992, 0.9998, 0.9997, 0.9993 and 0.9998 respectively for all these cardiovascular drugs. The values of RSD and confidence levels were in the range of 0.323-0.722% and 98.277-100.964% across the concentration ranges studied.

3.3.2.2.2 Limits of Detection and Quantitation

The LOD and LOQ of amiloride HCl, metoprolol tartrate, hydrochlorothiazide, carvedilol, amlodipine besilate, frusemide, telmisartan, losartan potassium and olmesartan were 0.2273 and 0.6886, 0.2285 and 0.6924, 0.3019 and 0.9147, 0.1674 and 0.5072, 0.3945 and 1.1955, 0.2015 and 0.6107, 0.2202 and 0.6671, 0.3559 and 1.0785, 0.2150 μgmL^{-1} and 0.6514 μgmL^{-1}, respectively.

3.3.2.2.3 Specificity

The method was quite specific as can be seen from Figure 3. The retention times of all molecules were almost similar in both standard solutions and plasma samples. There was no affect of the added impurities in the standards on the retention times and peak shapes of these molecules. These findings indicated good specificity of the reported method.

3.3.2.2.4 Precision

Precision data was calculated at three different concentrations i.e. 0.001, 0.005 and 0.025 $mgmL^{-1}$ of all the reported drugs. Five sets of the chromatographic runs were carried out for all three concentrations. The values of RSD and confidence levels were in the range of 0.323-0.722% and 98.277-100.00%.

3.3.2.2.5 Accuracy

Accuracy of HPLC method was ascertained using different concentrations of the reported molecules. Three concentrations used were 0.001, 0.005 and 0.025 $mgmL^{-1}$. The chromatographic runs were carried out five times (n = 5). The accuracies were determined by interpolation of five replicates peak areas of these molecules. The values of absolute errors were ranged from 0.1.5-0.5.

3.3.2.2.6 Robustness

Robustness of HPLC method was determined by changing a slight variation in the chromatographic experimental conditions. The varied experimental conditions were mobile phase composition, flow rate, temperature and wavelength. The retention time, peak area and shape were analyzed under the established and slightly varied experimental conditions.

3.3.2.2.7 Ruggedness

Ruggedness of the method was determined by changing the experimental conditions such as several operators and different days.

3.4. APPLICATION OF THE DEVELOPED SPE-HPLC METHODS TO THE REAL WORLD SAMPLES:

The developed and validated SPE and HPLC methods were applied for monitoring the reported drugs into human plasma samples. It was observed that there was no extra peak in the chromatograms (Figure 3), indicating good selectivity of SPE method. Besides, the absence of any extra peak confirms no new molecule and metabolic product during combination therapy. The HPLC results in terms of retention, separation, resolution factors and symmetry of the eluted peaks were similar to those of the standard samples. These findings showed that the

reported SPE and HPLC methods were selective, efficient, rugged and reproducible.

3.5. CONCLUSION:

The reported SPE and HPLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of amiloride, metoprolol, hydrochlorothiazide, carvedilol, amlodipine, frusemide, telmisartan, losartan and olmesartan in human plasma. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported cardiovascular drugs. Besides, the absence of any new peak established no metabolic product. The separation and identification of these nine cardiovascular drugs is reported first time so far. Therefore, SPE and HPLC methods can be applied for analyses of these nine cardiovascular drugs. The developed SPE and HPLC methods were applied successfully for monitoring these drugs into human plasma.

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4.1. INTRODUCTION:

The diabetes mellitus is a group of metabolic ailments involving high blood sugar. About 387 million people have been reported diabetes worldwide in 2014 [1-3]. Furthermore, 1.5 to 4.9 million deaths has been described from 2012 to 2014. It was estimated that the number of diabetic patients may increase up to 592 million by 2035. In this way, the world economy is affecting greatly. The affect on economy was estimated and found to be 245 billion US $ in 2012 [4-5] and 612 billion US $ in 2014 in USA alone [6]. Due to large number of the deaths and the economic pressure, the various drugs are being used to control the diabetes. The most important drugs are metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide (Figure 4.1). These drugs control diabetes by the different mechanisms.

In spite of the curing properties of these drugs, several side effects have also been reported [7-12]. Generally, these drugs are prescribed in the form of the combination therapy. Sometimes, this therapy may lead some serious side effects owing to the formation of the other species in the human body. Interestingly, the pharmacokinetics and pharmacodynamic of these molecules work in the different ways and differ combination therapies. As a result, there is an immense requirement to develop accurate sample preparation and analytical methods.

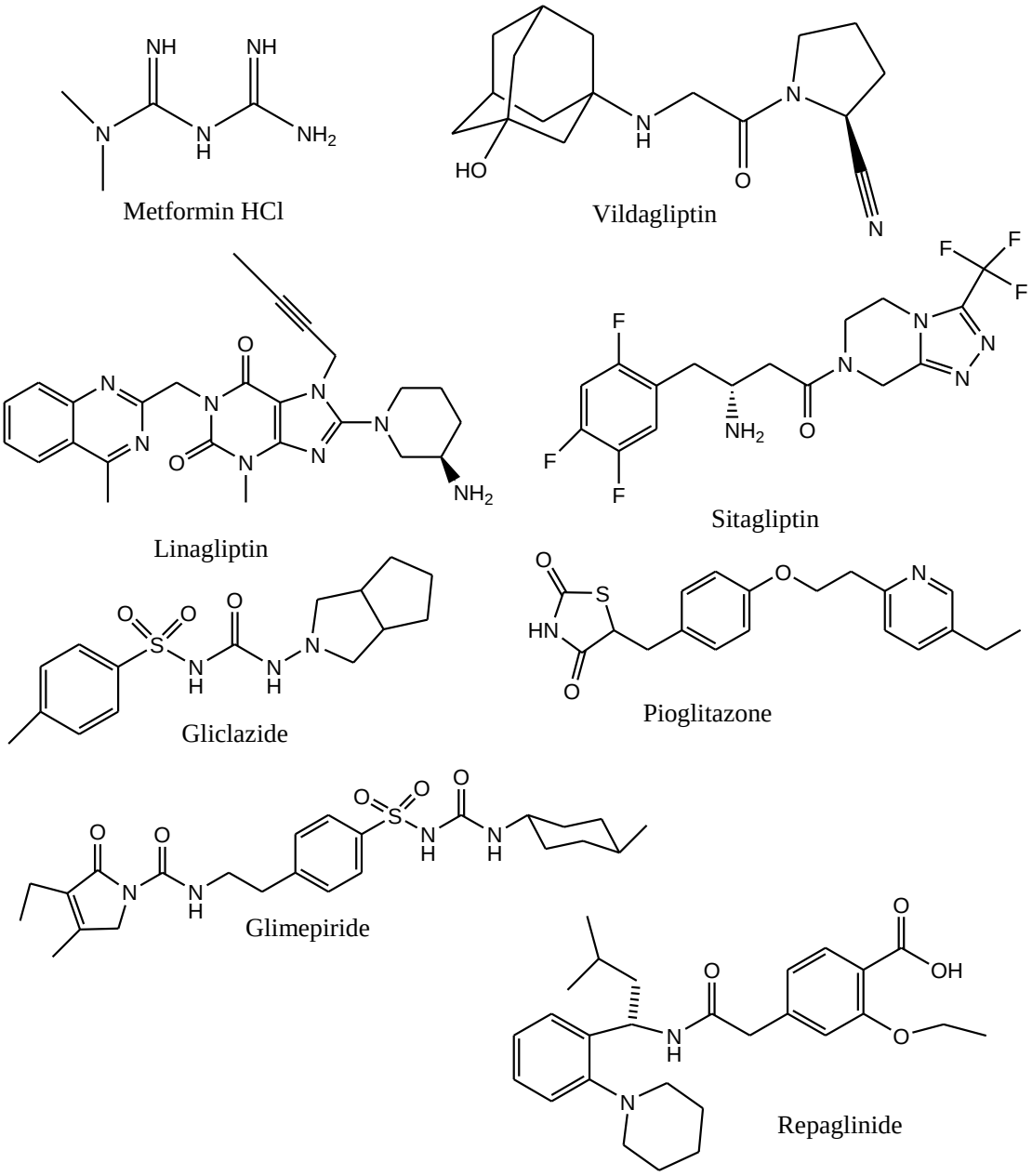

Figure 1:The chemical structures of antidiabetic drugs

4.2. LITERATURE SURVEY:

The separation and identification includes the chromatographic methodology used to identify anti-diabetic drugs. These analysis and separation are done by taking the advantage of difference in their properties [13]. The separation and identification of anti-diabetic drugs are carried by extraction and separation techniques. The most commonly used techniques are solid phase extraction (SPE) and high performance liquid chromatography (HPLC). A thorough search of literature on the analyses of anti-diabetic drugs was carried out through Scifinder, Scopus, and Science direct and peer reviewed Journals. This is discussed in the following sub-sections.

4.2.1 Metformin

Metformin is used for the treatment of type 2 diabetes, specially in people having overweight. It is also used in the treatment of polycystic ovary syndrome. Limited evidence suggests metformin may prevent the cardiovascular disease and cancer complications of diabetes. It is not associated with weight gain. Of course it is well tolerated but the known side effects such as diarrhea, nausea and abdominal pain. It has a low risk of causing low blood sugar. High blood lactic acid level is a concern if the drug is prescribed inappropriately and in overly large doses. It should not be used in those with significant liver disease or kidney problems. While no clear harm comes from use during pregnancy, insulin is generally preferred for gestational diabetes. Metformin is in the biguanide class and works by decreasing glucose production by the liver and increasing the insulin sensitivity of body tissues. It is on the World Health Organization's List of Important Medicine made

by Organization, the most effective and safe medicine needed in a health system. Metformin, believed to be the most widely used medication for diabetes is taken by mouth [12-14].

Alshishani et al. [15] defined an easy process of sample preparation, ion-pair vortex helped liquid liquid microextraction, for the metformin analysis, a extremely polar anti-diabetic medicine in sample of plasma was gave. The effects of ion-pair concentration, pH, volume of extraction phases, type of organic solvent, ionic strength, centrifugation time and vortexing on the extraction efficiency were examined. The best surroundings were at pH 9.3, 2 min centrifugation and 60s vortexing. The buformin (internal standard) microextract and metformin was directly injected into a HPLC unit by means of C_1 stationary phase and noticed at 235nm. The exactness was indoors 94.8−108% of the nominal concentration. The relative standard unorthodoxy for inter- and intra-day meticulousness was < 10.8%. The process was handily useful for the metformin analysis in sample of plasmas. Vaingankar et al. [16] discussed an easy reversed-phase HPLC process was for Metformin hydrochloride analysis in combination and estimation of their principal degradation products. The analysis was attained using JASCO Finepak SIL (250 mm × 4.6 mm i.d. 5 μm) at ambient temperature. The optimized eluent composed of an aqueous phase (pH 3.0 adjusted 20 mM phosphate buffer) and an organic phase (MeOH:ACN; 62.5 : 37.5) in 80:20 ratio. At 1.0 mL min^{-1} flow rate, the analytes were noticed at 230 nm. The given process was authorized for specificity, exactness, precision, sensitivity, and linearity. Gite and Patravale [17] discussed a constancy showing HPLC procedure for the metformin analysis HCl in mixture and

their main dilapidation products. The quantization and analysis were attained on a 5 µm Qualisil gold, C_{18} stationary phase. Phosphate buffer (pH 2.9) and organic phase (70 : 30) were selected as an eluent. Organic phase composed of MeOH-ACN (90:10, v/v) was run at 1.0 mL min^{-1} flow rate and recognition of analytes was carried out at 230 nm. Finally, the applicability of the process was gaged in nanoparticle medicine analysis.

Chhetri et al. [18] defined an easy HPLC-UV process for the metformin analysis in plasma of human. Ion pair analysis followed by UV recognition was done on deproteinized plasma of human samples. The analysis was carried out on a Discovery RP C_{18} stationary phase with UV recognition at 233 nm. The eluent contained 66% aqueous phase and 34% ACN. 10 mM KH_2PO_4 and 10 mM sodium lauryl sulfate were contained in aqueous phase. Aqueous phase pH was adjusted to 5.2. The eluent was run isocratically. The eluent flow rate was maintained at 1.3 mL min^{-1}. The linearity of the calibration curve was gotten in the concentration range of 0.125-2.5 µg mL^{-1} and coefficient of analysis (r2) was found to be 0.9951. The lowest limit of quantification and recognition was 125 and 62 ng mL^{-1}, correspondingly. The intra- and inter- days coefficient of variations was 6.97% or less for all the selected amounts. Ramesh and Habibuddin [19] discussed a easy, rapid, and precise RP-HPLC process for metformin analysis hydrochloride in bulk and its pharmacological formulations. These medicines were separated by using Grace Smart Altima C_8 stationary phase with an eluent consisting of ACN : phosphate buffer (60 : 40 (v/v), pH 3.0) at 1 mL min^{-1} flow rate, injection volume 25 µL, and recognition at 235 nm.. The process was authorized for linearity,

precision, exactness, specificity, and sensitivity in accordance with ICH (Q2B) guidelines. The results of all the proof parameters were found to be within the acceptable limits. Li et al. [20] defined a subtle, discriminating and quick HPLC-MS-MS process for the determination of metformin in plasma of human using phenacetin as internal standard (IS). After one-step precipitation of protein of 200 µL plasma with MeOH, metformin, was separated on a Kromasil Phenyl stationary phase (4.6 x 150 mm, 5 µm) at 40°C with an isocratic eluent consisting of MeOH-10 mM CH_3COONH_4 (75:25, v/v) at flow rate of 0.35 mL min^{-1}. The linear calibration curves were gotten over the amount choice 4.10-656 ng mL^{-1} for metformin. After oral administration, the presented HPLC-MS-MS procedure was proved to be fit for the kinetic study of metformin HCl tablets in healthy volunteers.

Pandit et al. [21] found an easy, precise, and accurate HPLC process for instantaneous estimation of metformin hydrochloride. Chromatographic analysis of the medicine was done by using a Phenomenex-ODS-3 C_{18} stationary phase with an eluent consisting of MeOH:ACN 15 mM potassium dihydrogen phosphate (pH 4) in the proportion of 40 : 35 : 25 (v/v) at 1.0 mL min^{-1} flow rate. Using a UV-SPD-10AVP detector at 240 nm, recognition was done.

Radwan et al. [22] found a UHPLC/MS assay for metmorfin analysis. For metformin amounts, the correctness (relative error) was <-10% and the intra- and inter days exactness (relative average deviation) values were <13%. After an oral dose at steady state, the test was subtle to follow the metformin kinetics in persons through a medicating intermission. Pharmacological constraints were calculated in 8 of these patients, and before and after their exposure to CM, metformin

accumulation was watched in 10 patients Sultana et al. [23] an easy, subtle, rapid, and accurate HPLC process for the analysis of in fresh materials, serum of human and their pharmacological formulations. In HPLC, medicine was chromatographed by means of pH (2.59 ± 0.02) and ACN-MeOH-H2O (30 : 20 : 50, v/v) as the eluent at 1.0 mL min^{-1} flow rate at ambient temperature. The analysis is carried out on a UV-vis detector equipped Hiber, 250-4.6 RP-18 stationary phase, at 230 nm. The process was accurate, precise and linear with apposite recognition and quantification limit. Fitness of the process for the quantitative medicine analysis was established by proof in agreement with the necessities laid down by Worldwide Meeting on Harmonization (ICH) guidelines.

4.2.2 Vildagliptin

Vildagliptin, a verbal anti-hyperglycemic agent (medicine of diabetes) of the new class of medicines of dipeptidyl peptidase-4 (DPP-4) which inhibit the inactivation of GLP-1 and GIP by DPP-4, allowing GLP-1 and GIP to potentiate the insulin excretion in the β cells and overturn glucagon release by the α cells of the Langerhans islets found in the pancreas. Adverse effects observed in clinical trials include nausea, hypoglycemia, tremor, headache and dizziness. Rare cases of hepatoxicity have been found. There have been case reports of pancreatitis associated with DPP-IV inhibitors. A group at UCLA found increased pre-cancerous pancreatic changes in rats and in human organ donors who had been treated with DPP-IV inhibitors. In response to these reports, the United States FDA and the European Medicine Agency each undertook independent reviews of all clinical and preclinical data related to the possible association of DPP-IV inhibitors

with pancreatic cancer. In a joint letter to the New England Journal of Medicine, the agencies stated that equally activities approve that statements concerning a fundamental connotation between incretin created medicines and pancreatitis or pancreatic tumour, as spoken newly in the systematic works and in the media, are unreliable with the present information.

De Andrade et al. [24] gave a new LC-MS-MS process for the vildagliptin quantification in a water dissolved solution. The process was positively authorized, meeting entirely the requisites of Medicine Administration guide & US Food for a bioanalytical process. The eluent employed was MeOH-CH_3COONH_4 5 mm (95 : 5). With diabetic male Wistar rats, a pharmacological experiment was conducted and the medicinal amount in liver was gaged by a microdialysis procedure. 50 mg/kg of the dose administrated was and the process permitted the vildagliptin quantification for >3 half lives, positively describing the pharmacological outline when the given process was useful. This was 1st report on the flesh pharmacologicals of a DPP-4 inhibitor and could contribute to medicine dose optimization in the impending. Satheesh kumar et al. [25] defined an easy, precise and constancy showing HPLC process for the analysis of vildagliptin in pharmacological quantity forms. The process involved the use of simply existing cheap test center reagents. On Grace Cyano stationary phase with isocratic flow, the analysis was attained. The eluent was pumped at 1.0 mL min^{-1} flow rate composed of 25 mM NH_4HCO_3 buffer and ACN (65 : 35, v/v). Over the concentration range of 50−250 µg mL^{-1}, a linear response was observed Limit of recognition and limit of quantification for MET were 0.75 µg mL^{-1} & 2.51 µgmL^{-1}.

The process was positively authorized in accordance to ICH guidelines acceptance criteria for system Fitness, linearity, specificity, exactness, precision and robustness.

4.2.3 Gliclazide

Gliclazide is an anti-diabetic medication used to treat diabetes mellitus type 2. Gliclazide was patented in 1966 and approved for medical use in 1972. It is on the World Health Organization's List of Essential Medicine, the most effective and safe medicine needed in a health system. It is used when dietary changes, exercise, and weight loss are not enough. The side effect may include low blood sugar, vomiting, abdominal pain, rash, and liver problems. The use by those with significant kidney problems, liver problems, or who are pregnant is not recommended. Gliclazide is in the sulfonylurea family of medications. It works mostly by increasing the release of insulin.

Sharma and Mazumdar [26] found HPLC process for gliclazide analysis, which was authorized according to ICH guidelines for system Fitness, exactness, linearity, sensitivity, precession, robustness & ruggedness. The limit of quantification (LOQ) & limit of recognition (LOD) were 0.03762 μg mL^{-1} & 0.11399 μg mL^{-1}, correspondingly. The observed values for number of theoretical plates (N > or = 2000), tailing factor (T < or = 2), asymmetry factor (AF < or = 1), and relative standard deviation (RSD < or = 1%) of useful process showed the reliability for gliclazide estimation in Isabgol husk hydrogel beads. Wang et al. [27] defined a discriminating and subtle liquid chromatographic-mass spectrometric (LC-MS) process for the gliclazide analysis in plasma of human. Sample handling

was founded on protein precipitation with ACN. With a triple quadrupole mass spectrometer, analytical analysis was approved out on a C_{18} stationary phase interfaced. 10 mM CH_3COONH_4, containing ACN−H_2O whose pH 3.5 adjusted with CH_3COOH (75 : 25) was used as the eluent was at 1.0 mL min^{-1} flow rate. In the concentration range of 0.025-2.5 µg mL^{-1}, the process was linear. The lower limit of quantification (LLOQ) was 0.025 µg mL^{-1}. The intra- and inter-day relative standard deviation across three proof runs over the entire concentration range was less than 9.8%. For gliclazide, the exactness determined at 3 amounts (0.05, 0.2 & 1.5 µg mL^{-1}) was within ±10.11% in relative error (RE) terms. The process defined was positively useful for the pharmacological profiles gliclazide evaluation sustained release tablets in 18 strong helpers. Mendes et al. [28] found a HPLC-MS/ MS for gliclazide analysis in human volunteers. The samples were extracted from plasma using diethylether : hexane (80 : 20, v/v) and the abstracts were analyzed by HPLC-MS-MS. Chromatography was done isocratically using a Genesis C_8 120A 4u. The process had a chromatographic run-time of 2.5 min.

Foroutan et al. [29] defined an easy, subtle and rapid isocratic RP HPLC process with UV recognition using a monolithic stationary phase has been authorized and given for gliclazide analysis in plasma of human. The assay enables the gliclazide measurement for therapeutic medicine monitoring with a least limit of quantification of 10 ng mL^{-1}. The process includes an easy, one step extraction method and analytical retrieval was comprehensive. The analysis was agreed in reversed phase surroundings using a Chromolith Performance stationary phase with an isocratic eluent consisting of 0.01M NaHPO4 buffer −ACN (52 : 48, v/v) whose

pH was adjusted to 4.0. At 230 nm, The λ_{max} was set. Over the concentration range 10.0 - 5000.0 ng mL^{-1}, the calibration curve was linear. The coefficients of variation for inter-day and intra-day assay were found less than 6.0%. Berecka et al. [30] defined a HPLC process for gliclazide quantitation in pharmacological formulations. Analysis was done using a LiChroCART RP-18 stationary phase, an eluent containing ACN-phosphate buffer (60 + 40, v/v pH 2.1), and ultraviolet (UV) recognition at 225 nm. Repaglinide was castoff as an internal standard for gliclazide analysis. The process was authorized *w.r.t.* precision, robustness, ruggedness, exactness, linearity, and specificity. The calibration graphs ranged from 0.015 to 0.09 mL min^{-1} for gliclazide. Intra- and interday relative standard deviation values for the standard solutions were 0.70 and 1.01% for gliclazide. Venkatesh et al. [31] found a HPLC process for gliclazide analysis in pharmacological formulation. On an Intertisl ODS 3V stationary phase, ternary gradient elution at 1.0 mL min^{-1} flow rate was employed at ambient temperature. The eluent composed of 0.01 m HCOOH (pH 3.0), ACN, Milli Q H_2O & MeOH. The plasma assay was authorized for parameters such as extraction recovery specificity and exactness. The process proposed was easy, discriminating and can be extended for routine analysis of anti-diabetics in biological matrices and in pharmacological preparations.

Kuo and Wu [32] discussed a subtle HPLC-electrochemical recognition process for the gliclazide analysis in plasma of human. By ACN deproteination of 100 μL of plasma, evaporation, and reconstitution, medicine was separated by the eluent (70 mM disodium tetraborate, pH 7.5, containing 26.5% of ACN) on a C_{18}

stationary phase. The limit of recognition for plasma was 10 nM for GL (S/N = 3, 10 microL booster). For monitoring blood levels with one healthy volunteer dosing with a GL tablet, this recently given process was useful. Park et al. [33] defined a chromatographic analysis with a semi-micro C_{18} stationary phase and 40 mM KH_2PO_4 (pH=4.6) – ACN - isopropyl alcohol (5 : 4 : 1, v/v) as the eluent, and with UV recognition at 229 nm. In the concentration range 0.1-10 μg/ml, calibration curve was linear. They were analogous, with no statistically significant difference when gliclazide pharmacological parameters in the two formulations were premeditated and compared statistically using crossover analysis of variance. This result suggests that when administered orally at a dose of 80 mg gliclazide, two formulations are bioequivalent. Rouini et al. [34] found rapid, an easy and specific process for gliclazide analysis in serum by a subtle HPLC. A little sample work-up Only 100 μL of serum and was required. An easy procedure of extraction by toluene followed by evaporation to aridity under a gentle stream of air and liquifying the dehydrated residue in mobile phase was used. The gliclazide peak was detached from endogenous peaks on a C_8 stationary phase by an eluent of ACN-H_2O (45 : 55, v/v), pH 3. Gliclazide and internal standard (phenytoin) were eluted at 6.8 and 3.8 min, correspondingly. Noguchi et al. [35] found an easy and subtle HPLC process for a routine gliclazide test in serum. The cartridge was eluted with 60% MeOH after washing with phosphate buffer (pH 7.5) and H_2O. The eluate was vanished to aridity. The residue was dissolved in MeOH and injected onto an octadecyl silica stationary. The eluent was 0.04 M potassium dihydrogenphosphate

(pH 4.6) – ACN - isopropyl alcohol (5 : 4 : 1, v/v). λ_{max} for Ultraviolet recognition was 227nm. The least noticeable level of gliclazide was 20 ng mL^{-1}.

4.2.4 Linagliptin

Linagliptin is a dipeptidyl peptidase-4 inhibitor given by Boehringer Ingelheim for diabetes mellitus treatment type 2. The side effect includes severe joint pain. Using pindolol as the internal standard, Hanafy et al. [36] defined a reproducible and subtle HPLC process for the linagliptin analysis in plasma of rat. Both the medicines were separated on a Zorbax Eclipse XDB C_{18} stationary phase kept at ambient temperature using eluent at 1.0 mL min^{-1} flow rate with a combination of HCOOH 0.1% 75% MeOH: 25% pH 4.1 at UV recognition of 254 nm. The assay was positively useful to an in vivo pharmacological study of the medicine in rats. Mourad et al. [37] found HPLC-DAD process for linagliptin quantitation in the occurrence of its dilapidation products in drugs and to get knowledge of its poverty kinetics. The medicine models were mined from diluted and tablets. Various ICH degradation surroundings were useful to study medicine degradation kinetics. The medicine was assayed by a C_{18} stationary phase using an easy isocratic eluent (pH 4.5 & MeOH : H_2O containing 0.3% TEA, 40:60,) pumped at 1.0 mL min^{-1}. λ_{max} to be noticed was 225 nm. With a total 17 min run time, the retention time was 11 min. In conclusion, easy, reliable and precise process for the analysis and medicine analysis in the presence of its degradation products under different stress surroundings was given & authorized according to the newest ICH guidelines. Vemula et al. [38] found a RP-HPLC process for linagliptin analysis. The present study aimed to develop an easy and subtle RP-

HPLC process for the linagliptin analysis in pharmacological dose forms. The chromatographic analysis was gaged and designed by using linagliptin working standard and sample solutions in the linearity range. Chromatographic analysis was done on a C_{18} stationary phase using an eluent of 70 : 30 (v/v) mixture of MeOH and 0.05 M KH_2PO_4 (whose pH was adjusted to 4.6 with H_3PO_4) delivered at 0.6 mL min^{-1} flow rate of with 267 nm UV recognition. In the range of 2-12 µg mL^{-1} with correlation co-efficient of 0.9996, Linagliptin linearity was osbserved. The resultant findings analyzed for standard deviation (SD) and relative standard deviation to validate the gave process. The process was authorized for exactness, linearity and precision were found to be acceptable over the linearity range of the linagliptin. The process was found fit for the routine quantitative analysis oflinagliptin in pharmacological dose forms. Jadhav et al. [39] defined a RP-HPLC, constancy showing process for linagliptin examination and its related substances in tablets. Using gradient elution and a detector wavelength of 225 nm, the analysis of nine specified impurities was attained at Zorbax SB-Aq stationary phase. The medicine was found to be forming degradation products during constancy studies and degrading extensively in heat, humidity, basic, and oxidation surroundings. The same process was used for liquid chromatography-mass spectrometry (LC-MS) and ultra-performance liquid chromatography -time-of-flight/mass spectrometry UPLC-TOF/MS analysis after slight modification in the buffer and the stationary phase,.

4.2.5 Sitagliptin

Sitagliptin, an enzyme inhibiting medicine is an oral antidiabetic medicine of class of the dipeptidyl peptidase-4 (DPP-4) inhibitor. This used either in

combination with other oral anti-hyperglycemic agents such as metformin or alone or a thiazolidinedione for diabetes mellitus treatment type 2. The side effects are as common with sitagliptin (whether used alone or with metformin or pioglitazone) as they were with placebo, except for rare nausea and common cold-like symptoms, including photosensitivity. No significant difference exists in the occurrence of hypoglycemia between placebo and sitagliptin. In those taking sulphonylureas, the risk of low blood sugar is increased. The existence of rare case reports of renal failure and hypersensitivity reactions is noted in the United States prescribing information, but a causative role for sitagliptin has not been established [40-43].

Ramalingam et al. [44] found a HPLC process for sitagliptin analysis in pharmacological dose form. The eluent composed of MeOH and H2O (70 : 30, v/v) with 0.2 % of n-heptane sulfonic acid whose pH was adjusted to 3.0 with H_3PO_4 was used. The stationary phase used was C_8 with 1.0 mL min^{-1} flow rate. Tang et al. [45] used an UPLC-MS-MS process for the sitagliptin analysis phosphate in plasma of rat. The blank plasma of rat sample added with sitagliptin phosphate and the internal standard (fluoxetine) standard solution were prepared. MeOH was added in the sample for the deproteinization. Then the sample was vortex-mixed and centrifuged. The clear supernatant was used for the analysis of UPLC – MS -MS. A Thermo Hypersil Gold C_{18} stationary phase was employed with a guard stationary phase of Phenomenex Security Guard C_{18} stationary phase and the stationary phase temperature was set at 35 ºC. The ramp elution of ACN and H_2O (containing 0.05% HCOOH) as eluents was done at 200 μL min^{-1} flow rate, and a rapid analysis was completed in 5 min. The mean recoveries were from 85% to 115% at the spiked

levels of 5, 50 and 500 μg/L; the relative standard deviations of inter- and intra-day of variation were both < 15%, which can meet the analysis requirements of biological samples. After the administration of a single intravenous injection dose of sitagliptin phosphate, the process was initially used for the sitagliptin analysis phosphate in SD plasma of rat. The process was rapid, subtle, convenient and reproducible in the sitagliptin analysis phosphate, and can be used for the pharmacologicals research of sitagliptin phosphate in plasma.

A zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) process was used by Rao et al. [46] for discriminating extraction and sitagliptin analysis in rat serum and urine. The effects of progenic solvents pH, monomer amount and cross linker were considered to enhance the efficacy and discrimination. The proposed process disables the phospholipids matrix effects generally encountered while sample of plasmas preparation by proteins precipitation. Nirogi et al. [47] gave a subtle HPLC-positive ion electrospray tandem mass spectrometry process for sitagliptin quantitative determination in plasma of human. With a relative average deviation of less than 6%, the lower LOQ was 0.1 ng mL^{-1}. Suitable meticulousness and exactness were gotten for amounts over the standard curve range. A run time of 2.0 min for each sample made it possible to analyze more than 300 plasma of human samples/day. The authorized process was positively used to analyze plasma of human samples for application in pharmacological studies.

4.2.6 Pioglitazone

Pioglitazone, a medicine of the thiazolidinedione class with hypoglycemic (antidiabetic, antihyperglycemic) is used to diagnose diabetes and it does decrease blood sugar levels, studies on the main cardiovascular outcomes have not yielded statistically significant results. Its circulatory safety outline associates favorably with that of rosiglitazone, which was withdrawn from some markets after anxieties around an increased cardiac events risk. Pioglitazone has been originated to be related with bladder cancers. It has been reserved in some nations [48-53].

Chinnalalaiah et al. [54] defined a liquid chromatography tandem mass spectrometry (LC-MS/MS) based process for pioglitazone estimation and its active metabolites in plasma of human for applicability to pharmacological studies. The chromatographic investigation was carried on the RP Peerless Basic C_{18}, stationary phase at temperature of 40°C using a binary eluent consisting of MeOH : 5 mM CH_3COONH_4 in 0.1% HCOOH (80 : 20, v/v). The sample injection was 10 μL and the eluent was run at 1.0 mL min^{-1} flow rate. The process utilized pioglitazone D4 and 5-hydroxyl pioglitazone M-IV D4 as an internal standards. The linearity of the process was authorized over the range of 6.04-1503.21 ng mL^{-1} for pioglitazone and 6.01-1496.28 ng mL^{-1} for 5-hydroxyl pioglitazone. The gave process was employed in healthy human volunteers to monitor the pharmacologicals profile of pioglitazone. In Wistar rats, Sharma and Pancholi [55] defined a RP-HPLC coupled with a diode array sensor for the pharmacological communication learning of atorvastatin with pioglitazone. The stationary phase used was C_{18} with an eluent composed of 48% MeOH, 19% ACN, and 33% 10 mM $HCOONH_4$ (v/v; pH

3.5±0.3, by HCOOH) and at 260 nm recognition wavelength on the diode array detector. The process was authorized according to international standards with good reproducibility and linear response; mean (r) 0.9987 and 0.9972. The coefficients of variation of intra- and interassay precision ranged between 4.95-8.12 and 7.29 - 9.67. Kawaguchi-Suzuki et al. [56] found an easy and rapid LC-MS-MS process was for the pioglitazone analysis and its lively metabolites ketopioglitazone and hydroxypioglitazone in plasma of human. With ACN and discriminating phospholipid depletion in a 96-well plate format, samples were processed by precipitation of protein. The process used deuterated internal standards for each analyte. Chromatographic analysis was attained on a Hypersil GOLD C_{18} stationary phase with gradient elution. With nonalcoholic fatty liver sickness taking pioglitazone, the authorized process was subtle and discriminating and positively useful to analyze clinical samples gotten from patients

Ni et al. [57] found a discriminating, rapid and subtle and LC-ESI-MS-MS for the estimation and pharmacological pioglitazone investigation in plasma of human. The chromatographic analysis was achieved using a eluent consisting of MeOH-H_2O-HCOOH (95 : 5 : 0.1, v/v, plus 5 mM CH_3COONH_4) at 1.0 mL min^{-1} flow rate of 0.8 on an Agilent Eclipse plus C_{18} stationary phase. The elution time was about 2.4 min and the total run time was 3.0 min. The process exhibited good analysis of analytes, without interference from endogenous substances. Peraman et al. [58] defined an easy, discriminating, precise, rapid and economical reversed-phase HPLC (RP-HPLC) process for pioglitazone hydrochloride estimation from pharmacological design. The process was carried out on a C_8 stationary phase with

an eluent consisting of ACN : H_2O (whose pH was adjusted to 6.2 using *o*-H_3PO4) in the ratio of 45:55 (v/v). The process was authorized in terms of exactness, linearity, specificity, precision, selectivity, limit of quantitation, limit of recognition and solution constancy. The proposed process was used for instantaneous estimation of these medicines in marketed dose forms. Musmade et al. [59] defined an HPLC process for the glimepiride analysis using C_{18} stationary phase and UV detector. An eluent composed of ACN and CH_3COONH_4 buffer pH 4.5 (55:45). The sample of plasmas clean-up was done using solid phase cartridges. The process was in the linear range of 50-8000 ng mL^{-1}. The given process was useful for preclinical pharmacological studies.

Arayne et al. [60] found a reverse-phase HPLC process, authorized & useful for the analysis pioglitazone hydrochloride in serum of human and tablets. Chromatographic analysis was attained on a C_{18} stationary phase with an eluent consisting of MeOH-H_2O-ACN (80 : 10 : 10, v/v) with 0.7 mL min^{-1} flow rate whose pH was adjusted to 3.50 with H_3PO4 at 230 nm. Internal standard was Glibenclamide. Thus, the proposed process was easy and fit for the instantaneous analysis of active ingredients in dose forms and serum of human. For the pioglitazone analysis in plasma of human, Islambulchilar et al. [61] defined an easy and quick HPLC process with UV recognition. Using perchloric acid on an ODS stationary phase, the process was based on precipitation of protein. The eluent composed of a mixture of phosphate buffer, MeOH, ACN, and 12 M $HClO_4$ (54 + 33 + 12 + 1, v/v). λ_{max} for UV detection was 269 nm. Under these surroundings, the pioglitazone retention time was 5.2 min. Over the range of 50 - 2000 ng mL^{-1}

pioglitazone in plasma of human, the standard curve was linear. The within-day and between-day precision studies showed high reproducibility, with CV < 5%. Tahmasebi et al. [62] defined a HPLC process for the pioglitazone analysis. In this procedure, the target medicine was extracted into di-n-hexyl ether restrained in the wall pores of a porous hollow fiber from 10 mL of the aqueous sample (source phase, SP) with pH 8.0, and then back extracted into the receiving phase (RP) with pH 2.2 located in the lumen of the hollow fiber. Due to a pH gradient between the two sides of the hollow fiber, the extraction occurred. 24 μL of the RP solution was taken back into the syringe and injected directly into a HPLC instrument for quantification after removing for a prescribed time. The % relative intra-day and inter-day standard deviations (RSDs%) based on five replicate analysiss were 4.7 and 15%, correspondingly.

4.2.7 Glimepiride

Glimepiride, sometimes classified as either the first third-generation sulfonylurea or as second-generation is an orally available medium-to-long-acting sulfonylurea antidiabetic medicine. Its mode of deed is to raise insulin manufacture by the pancreas. Glimepiride is showed to treat type 2 diabetes mellitus. It is not used for type 1 sugar disease because in type 1 diabetes the pancreas is not able to produce insulin. The side effects of glimepiride include gastrointestinal tract disturbances, occasional allergic reactions, and rarely blood production disorders including thrombocytopenia, leukopenia, and hemolytic anemia. In the initial weeks of treatment, the risk of hypoglycemia may be increased. Alcohol

consumption and exposure to sunlight should be restricted because they can worsen side effects [63-67].

Surendran et al. [68] defined a RP HPLC processs having RP stationary phase with eluent consisting of ACN and 10 mM CH_3COONH_4 (pH-6.0) in the ratio 85 : 15 (v/v) at 1.0 mL min^{-1} drift rate for the chromatographic analysis. LLOQ was found to be 5 ng mL^{-1} for both PIT and GLI and a total run time of 3.0 min. The process was useful to study the medicine interaction between GLI and PIT in rat liver microsomes. Dewani et al. [69] found a novel, easy and mass spectrometry (MS) compatible HPLC process for the glimepiride estimation in plasma of rat. The bio-analytical procedure involves extraction and liquid chromatography. The chromatographic analysis was done on H_2Os-600 system using an isocratic eluent comprising MeOH:H_2O (80 : 20 % v/v) at 1.0 mL min^{-1} flow rate with pH of H_2O modified to three using HCOOH and Kinetex C_{18} stationary phase maintained at 30 ± 1°C. The process was positively useful in pharmacological studies followed by oral administration of the medicine. Qiu et al. [70] used a subtle and quick UPLC-MS-MS process to determine glimepiride in plasma of human. On an Acquity UPLC BEH C_{18} stationary phase, chromatographic analysis was carried out and mass spectrometric analysis was done using a QTrap5500 mass spectrometer coupled with an electro-spray ionization (ESI) source in the positive ion mode. This assay process was fully authorized in selectivity, recovery, linearity, and exactness, matrix effect, precision and constancy terms. Within the concentration range of 2.5-500 ng mL^{-1} in plasma of human, the linearity of this process was observed. For an analytical run, only 1.5

min was needed. This assay was used to support a clinical study where multiple oral doses were administered to healthy Chinese subjects to examine the medicine pharmacologicals.

Bonfilio et al. [71] defined the multivariate development of a constancy-indicating HPLC process for the glimepiride quantification in pharmacological tablets. Full factorial design, Doehlert design, and response-surface processology were used in conjunction with the desirability function approach. This procedure allowed the adequate glimepiride analysis from all degradant peaks in a short analysis time. This HPLC process uses K_3PO_4 buffer (pH 6.5; 27.5 mmol/L)-MeOH (34 + 66, v/v) eluent at 1.0 mL min^{-1} flow rate and UV recognition at 228 nm. A H_2Os Symmetry C_{18} stationary phase at controlled room temperature (25 degrees C) was used as the stationary phase. The process was authorized affording to International Conference on Harmonization guidelines & demonstrated linearity from 2 to 40 mg/L glimepiride, selectivity, precision, exactness, and robustness. The LOQ and LOD were 1.050 and 0.315 mg/L, correspondingly. The multivariate strategy adopted was positively useful in routine laboratories because of its fast optimization without the additional cost of stationary phases or equipment. Tripathi et al. [72] used an easy and subtle process for the glimepiride estimation in plasma of rat by RP-HPLC. By liquid-liquid extraction with 300 µl of ACN and 5 ml of diethyl ether, the medicine models were extracted. Chromatographic analysis was attained on C_{18} stationary phase with UV recognition at 230 nm using MeOH : H_2O (85 : 15 v/v) as eluent at 1.0 mL min^{-1} flow rate. The given process was authorized for exactness linearity, precision, and recapture. Over the range of 100 - 12000 ng

mL^{-1}, the process was linear and found to be suitable. The process was positively useful for the analysis of plasma of rat model for application to pharmacological. El-Enany et al. [73] defined an easy subtle HPLC process for the medicine analysis in their combined dose forms and plasma. The analysis was attained using a Symmetry® C_{18} stationary phase. At 1.0 mL min^{-1} flow rate, the eluent containing a mixture of ACN and 0.02 M phosphate buffer of pH 5 (60: 40, v/v) was pumped. λ_{max} for UV recognition was 235 nm. The process was authorized for specificity, precision, linearity, sensitivity and exactness. The suggested process was positively useful for the considered medicine analysis in their co-formulated tablets and plasma of human.

Chakradhar et al. [74] found a subtle and specific liquid chromatography-positive electrospray ionization-tandem mass spectrometry process the glimepiride analysis in plasma of human. On a YMC Propack, C_{18}, stationary phase, the mixtures were analysed using CH_3COONH_4 buffer, ACN and MeOH (30 : 60 : 10, v/v) as eluent at 0.5 mL min^{-1} flow rate on an API 4000 Sciex mass spectrometer connected to an Agilent HPLC system. pre-clinical sample analysis and process proof was done as per FDA guidelines and the results met the acceptance measures. El Deeb et al. [75] defined a fast process for the instantaneous analysis and glimepiride analysis by RP-HPLC on a Chromolith Performance stationary phase. As eluent, a mixture of phosphate buffer pH 3, 7.4 mM, and ACN (55 : 45 v/v) was used. Stationary phase oven temperature was established to 30 ºC. The exactness of the intra-day and the inter-day assay for both retention times and peak areas for the 4 investigated compounds were < 1.2%. The process displayed moral recovery &

linearity. The analysis in short time made the process very respected for quality rheostat and constancy testing of medicines and their pharmacological measures. Using UV recognition for the glimepiride analysis from plasma of human samples, Song et al. [76] gave a fully automated semi-microbore high performance liquid chromatographic (HPLC) process with stationary phase-switching. Sample of plasma extracted with EtOH and ACN and (900 μl) was deproteinated.

4.2.8 Repaglinide

Repaglinide, an antidiabetic medicine in the class of medications known as meglitinides was invented in 1983. Repaglinide is an oral medication used in addition to exercise for blood sugar control and diet in type 2 diabetes mellitus. The of action mechanism of repaglinide involves promoting insulin release from pancreas β-islet cells; like other antidiabetic medicines, a main side effect concern is hypoglycemia [77].

Chen et al. [78] defined a completely robotic auto-sampler/HPLC-MS/MS scheme for unattended droplet-based surface sampling of repaglinide dosed thin tissue pieces with subsequent HPLC analysis and mass spectrometric parent medicine analysis. A semi-quantitative agreement between data gotten by employing organ homogenate extraction and that by surface sampling was detected. Dhole et al. [79] found reversed-phase high performance liquid chromatography (RP-HPLC) process for repaglinide analysis in the tablet dose form. RP-HPLC analysis was done using an eluent composed of MeOH and H_2O (80 : 20 v/v, pH adjusted to 3.5 with *o*-H_3PO_4) at 1.0 mL min^{-1} flow rate on Agilent TC-C_{18} stationary phase. The given process was found to be reliable, easy, fast, accurate

and positively used for the quality control of repaglinide as a bulk medicine and in pharmacological formulations. Jirovský et al. [80] defined an easy, fast and subtle HPLC process for the repaglinide analysis in plasma of human. The eluent composition was 50 mM Na_2HPO_4-ACN (60 : 40, v/v), pH of the eluent 7.5 adjuseted with H_3PO_4. Over the concentration range of 5 - 500 nmol/L, Linear Calibration curve was observed. The limit of recognition (LOD) was established at 2.8 nmol/L, and the lower limit of quantification (LLOQ) at 8.5 nmol/L. Rosiglitazone was used as an internal standard. The given process was useful to plasma of human samples spiked with repaglinide at the rapeutical amounts. It was established that the process was fit for pharmacological trainings or relaxing monitoring.

Kaushal et al. [81] found a HPLC process for repaglinide analysis. The process was authorized for repaglinide analysis in pills and in receptor fluid gotten during in vitro permeation trainings. HPLC repaglinide analysis yielded retention time of 6.14 min with linearity ranging from 0.1-1.2 μg mL^{-1} concentration. Al Khalidi et al. [82] defined a fast and reliable process for the repaglinide analysis to support formulation screening and quality control. Additionally, the process was positively used for the dissolution test of repaglinide and was found to be reliable, easy, fast, and inexpensive. Ruzilawati et al. [83] gave a HPLC assay for repaglinide analysis concentration in plasma of human for pharmacological trainings. Sample of plasma containing an internal and standard repaglinide indomethacin were extracted with ethylacetate at pH 7.4. Chromatographic analysis was done on Purospher STAR C_{18} analytical stationary phase. The eluent was

composed of ACN-$HCOONH_4$ (0.01 M; pH 2.7) (60:40, v/v) at 1.0 mL min^{-1} flow rate. Repaglinide and indomethacin retention times were approximately 6.2 and 5.3 min, correspondingly. This process was useful for the determination of repaglinide amount in plasma of human samples for a pharmacological study. Gandhimathi et al. [84] found an easy, rapid and precise RP-HPLC process for the repaglinide analysis in pharmacological dose forms. Using nimesulide as internal standard, the process was done on a Shim-pack, RP-C_{18} stationary phase using a combination of MeOH : 0.1% v/v triethylamine (whose pH was adjusted to 7 with ortho-H_3PO_4) and λ_{max} for recognition was 235 nm.

As indicated above, a thorough search of prose was carried out using Scifinder, peer reviewed, Scopus and Science direct Journals. It was observed that there are number of HPLC processs for the analyses of these medicines but nobody defines UFLC analysis of all these molecules instantaneously on sketchily porous silica particles (core shell technology). As well, only rare papers pronounce processs of sample preparation. Moreover, it was realized that these processes are time and costly chemical consuming. Moreover, the limits of recognitions are quite high. With trust in these facts, the exertions were made to confirm and develop solid phase extraction (SPE) and ultra-fast liquid chromatographic (UFLC) processs for the analysis of these medicines in the plasma of human. The results of these tests are discussed herein.

4.3. RESULTS AND DISCUSSION:

The outcomes and argument is divided into 2 parts viz. UFLC and solid phase extraction. These are discussed in the following sub-sections.

4.3.1 Solid Phase Extraction

Solid phase extraction process was applied to detached these medicines from the sample of plasmas. The percentage salvages of each medicine were premeditated. The percentage recoveries of repaglinide, gliclazide, glimepiride, pioglitazone, metformin HCl, linagliptin, vildagliptin, and sitagliptin were determined by doing the outright experiments. The intended percentage recoveries of these medicines in the laboratory synthesized samples in H2O and plasma are given in Table 4.1. An examination of this table shows that the % recovery values of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide in laboratory synthesized samples in H_2O were 80, 82, 77, 87, 83, 85, 86, and 88%, correspondingly. These values in plasma were 22, 20, 21, 19, 16, 12, 10, and 17%, correspondingly. Low values in the sample of plasmas were due to the requisite of these medicines with plasma proteins. The best settlement of SPE was gotten by varying the different SPE surroundings viz. amounts and pHs of phosphate buffer and plasma sample plasmas flow rates, eluting solvents and phosphate buffer. The additional eluting solvents viz. EtOH, MeOH, ethyl acetate and dichloromethane were also used. Besides, some acid additives such CH_3COOH, trichloro CH_3COOH and trifluor CH_3COOH were also castoff. Therefore, the best eluting solvent was MeOH with 0.1% CF_3COOH. It was understood that the polarity of MeOH was enlarged by totaling CF_3COOH,

resulting in good elution of these medicines from C_{18} cartridge. The maximum percentage recoveries of these medicines were gotten by phosphate buffer (20.0 mM, pH 7.0), independently and correspondingly, at the flow rate of 0.1 mL min^{-1}. The values of RSD, correlation coefficient (R) and confidence level for these drugs were ranged from 1.4 to 1.8, 0.9993 to 0.9994 and 99.0 to 99.3, correspondingly (Table 4.2).

Table 4.1. The percentage recoveries of antidiabetic drugs in the laboratory synthesized samples in water and human plasma samples using solid phase extraction.

S.No.	Name of drugs	% Recovery in water	% Recovery in human plasma
1.	Metformin HCl	81	21
2.	Vildagliptin	83	22
3.	Gliclazide	78	20
4.	Linagliptin	88	20
5.	Sitagliptin	84	17
6.	Pioglitazone	86	13
7.	Glimepiride	87	11
8.	Repaglinide	87	18

Table 4.2. Validation data of SPE method for antidiabetic drugs.

S.No.	Drugs	%RSD	Correlation Coefficient (r)	Confidence Level (%)
1.	Metformin HCl	1.6	0.9993	99.3
2.	Vildagliptin	1.7	0.9994	99.4
3.	Gliclazide	1.3	0.9994	99.2
4.	Linagliptin	1.4	0.9994	99.5
5.	Sitagliptin	1.7	0.9995	99.6
6.	Pioglitazone	1.4	0.9994	99.6
7.	Glimepiride	1.4	0.9994	99.7
8.	Repaglinide	1.6	0.9993	99.5

4.3.1.1 Optimization

As usual solid phase extraction is the best tool for extraction of various molecules. The same method was applied for extraction of cardio anti-diabetic drugs in human plasma. The percentage recoveries of anti-diabetic drugs from standard samples ranged between 77 to 88%, respectively. These values were from 10 to 22% in plasma samples. These results indicated that solid phase extraction was an optimum method for the extraction of cardio vascular drugs. The recoveries were less in plasma samples as compared to standard samples because in case of plasma samples some amount of anti-diabetic drugs interacted with the protein molecules and cannot be extracted by SPE. It is also interesting to observe that in HPLC chromatograms no extra peaks were found, which shows the selectivity of the solid phase extraction method. SPE process was also optimized by pH of the plasma, flow rate of plasma and flow rate of eluting solvent. Besides, various eluting solvents *viz*. methanol, dichloromethane, ethanol, acetone and ethyl acetate

were used for the optimization of the solid phase extraction method. An extensive experimentation was carried out for optimizing SPE conditions and the experimental procedure is reported in the following sections.

4.3.1.1.1 Effect of pH of Plasma

The percentage recoveries of the analytes change according to the pH of the plasma. In solid phase extraction, the purification of the analyte from the plasma occurs on the basis of adsorption on the silica gel of the C_{18} cartridge. This process of adsorption is mainly governed by the adjustment of plasma pH. The pH range from 1.0 to 10.0 was used in this extraction process, to find out the variation in percentage recoveries with the change in the pH of the plasma. The Figure 4.2 shows the effect of various pH ranges on the percentage recoveries of anti-diabetic drugs. It is clear from the figure that at lower pH values the percentage recoveries are poor and increased up to 7.0 pH. Further increase in pH also could not increase the extraction percentage recoveries in the standard solutions. The maximum percentage recoveries of the drugs at pH 7.0 were in the range of 77-88%. The reason of the various values at pH 7.0 is due to different ionic strength of the drugs.

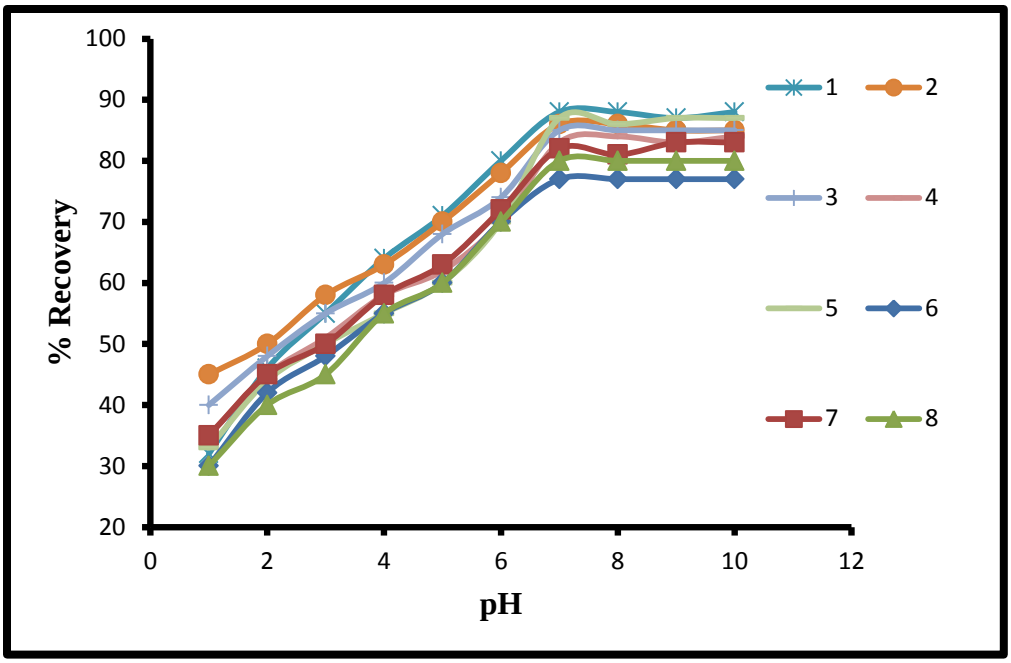

Figure 4.2: The effect of different pH of plasma on the percentage recoveries of anti-diabetic drugs.

1. **Metformin HCl, 2. Vildagliptin, 3. Gliclazide, 4. Linagliptin, 5. Sitagliptin, 6. Pioglitazone, 7. Glimepiride and 8. Repaglinide.**

4.3.1.1.2 Effect of Flow Rate of Plasma

The run rate is one of the most significant optimizing variables in solid phase extraction. Usually, elevated percentage revivals of the molecules are attained at less run rate of plasma while quick run rate guides to the reduced percentage revivals of the molecules. Because of this, efforts were made towards the proper optimization of the solid phase extraction for maximum recoveries of the drugs. For this purpose different run rates such as 0.025, 0.05, 0.075, 0.10, 0.125 and 0.15 mL min^{-1} were tried in the extraction process. The outcomes of the experiments are exposed in Figure 4.3 and this figure clearly demonstrates that percentage recoveries of the anti-diabetic drugs ranged from 77 to 88 at different flow rates. Of course, the highest percentage revivals were at 0.025 mL min^{-1} flow

rate but this flow rate was too low to use experimentally. Keeping all the facts, into consideration, 0.10 mL min^{-1} flow rate was considered worthwhile and the maximum percentage recoveries at this flow rate were 77 to 88 percent. Therefore this flow rate was used throughout this study.

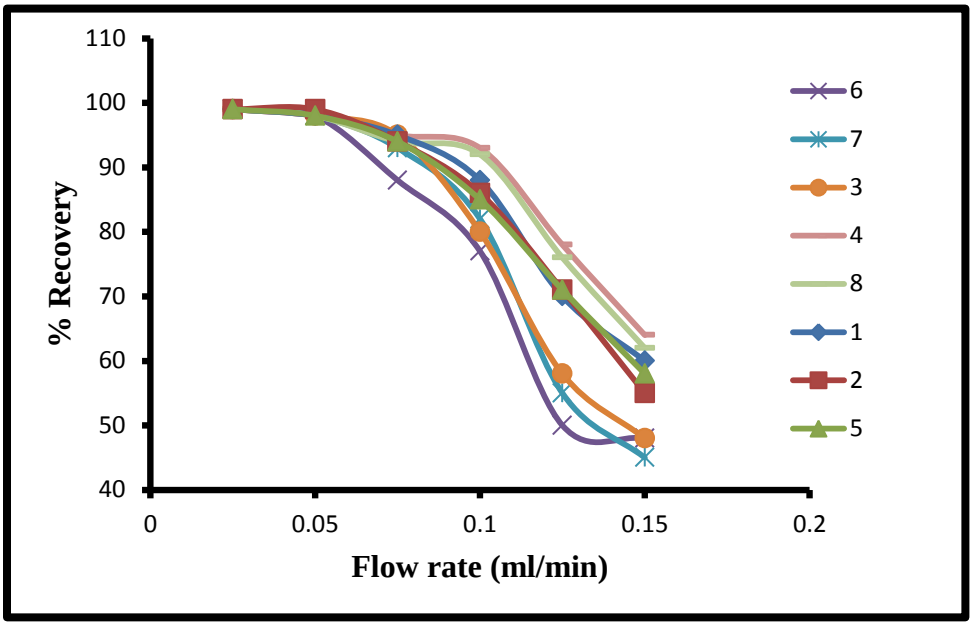

Figure 4.3: The effect of different flow rates of plasma on the percentage recoveries of anti-diabetic drugs.

1. **Metformin HCl, 2. Vildagliptin, 3. Gliclazide, 4. Linagliptin, 5. Sitagliptin, 6. Pioglitazone, 7. Glimepiride and 8. Repaglinide.**

4.3.1.1.3 Effect of Other Solvents

The selection of suitable eluting solvent also governs the optimization of the solid phase extraction. So, in order to investigate the best eluting solvent for the elution of essential amino acids through the C_{18} cartridge various solvents were tried. MeOH, CH_2Cl_2, EtOH, CH_3COCH_3 and $CH_3COOCH_2CH_3$ were used as the eluting medium to obtain the maximum recoveries of the anti-diabetic drugs from the C_{18} cartridge. The percentage recoveries of the drugs obtained by the use of these five solvents are presented in the Figure 4.4. It is very clear from this figure that the order of percentage recoveries of the analytes is MeOH > CH_2Cl_2> EtOH > CH_3COCH_3 > $CH_3COOCH_2CH_3$. A critical evaluation of this set of experiment indicated that the highest and least percentage revivals of the drugs were attained by using methanol and ether, respectively. Consequently, it was accomplished that methanol was the best eluting solvent for the maximum recoveries of the drugs through the C_{18} cartridge. Different polarities and dielectric constant values of these solvents are responsible for their different behavior. Amid these solvents MeOH was extra capable in the procedure of desorption of the drugs via the C_{18} cartridge as it had superior dielectric constant magnitude and polarity than that of others. Besides MeOH, CH_2Cl_2 also had substantial magnitudes of dielectric constant and polarity and, thus, also gives good percentage recoveries of the drugs. But the use of methylene dichloride was avoided in the experiments because of its volatile nature, which is not safe for the experimental operator. And for the remaining solvents the magnitudes of dielectric constants and polarities were not important to reason the bond dissociation between the drugs and C_{18} material of solid phase

cartridge. Due to which they gave low values of percentage recoveries of the reported drugs. Consequently, MeOH was established to be the most excellent eluting solvent and it was utilized all through the experimentation.

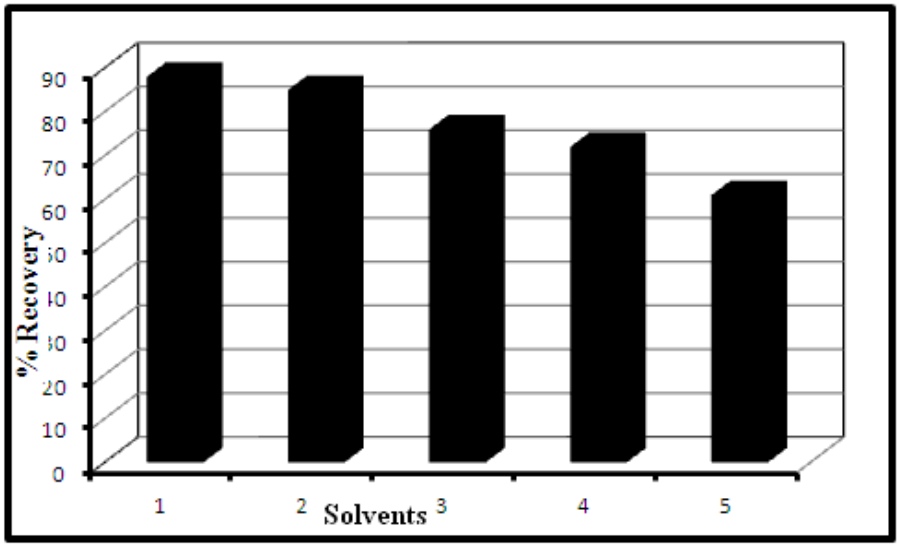

Figure 4.4: The effect of different solvents on the percentage recoveries of anti-diabetic drugs.

1. **Methanol, 2. Dichlorometane, 3. Ethanol, 4. Acetone, and 5. Ethyl acetate.**

4.3.2 Ultra Fast Liquid Chromatography

The ultra fast liquid chromatographic parameters viz. retention (k), separation (α), and resolution (Rs) factors were determined for metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide drugs. The values of these UFLC parameters are given in Table 4.3. The values of the retention, separation and resolution factors were ranged from 0.07 to 9.14, 1.44 to 4.21 and 2.15 to 18.66, correspondingly. The UFLC chromatograms of these drugs in standard and plasma samples are reported in Figure 4.5 and 4.6,

respectively. It is apparent from this figure that the described drugs were base line separated. The identification of the separated drugs was determined by running and comparing the retention times of the individual metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide molecules, correspondingly. It was observed that there was no additional peak in the plasma samples, which established the selectivity of the SPE method.

Table 4.3: Chromatographic parameters i.e. capacity factors (k), separation factors (α), resolution factors (Rs) of antidiabetic drugs.

Sl. No.	Antidiabetics	(k)	(α)	(Rs)
1.	Metformin HCl	0.072	-	-
2.	Vildagliptin	0.301	4.208	2.147
3.	Linagliptin	0.555	1.843	2.223
4.	Sitagliptin	0.828	1.493	2.325
5.	Gliclazide	1.195	1.443	3.039
6.	Pioglitazone	3.114	2.607	13.080
7.	Glimepiride	3.836	1.232	3.970
8.	Repaglinide	9.140	2.383	18.652

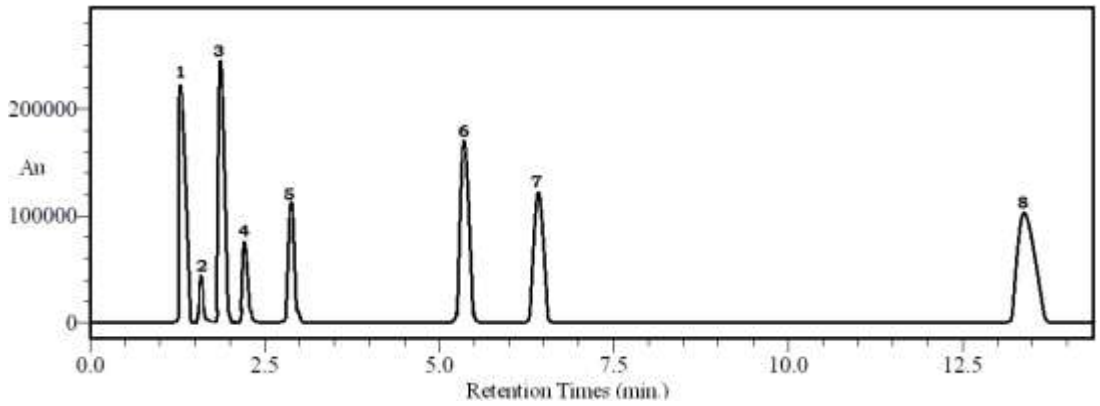

Figure 4.5: UFLC chromatogram of antidiabetic drugs in standard solutions.

1. **Metformin HCl, 2. Vildagliptin, 3. Gliclazide, 4. Linagliptin, 5. Sitagliptin, 6. Pioglitazone, 7. Glimepiride and 8. Repaglinide.**

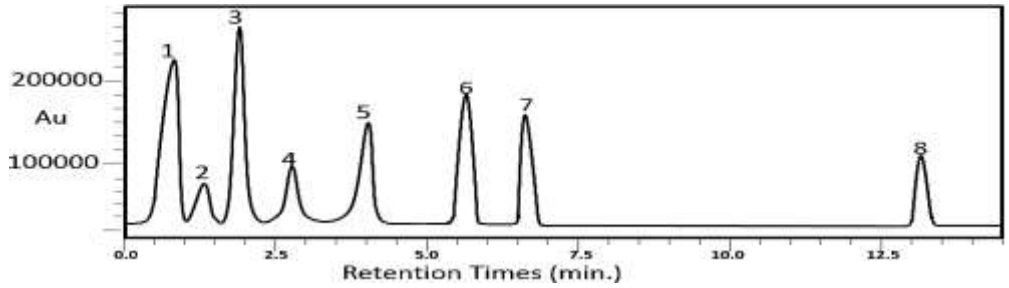

Figure 4.6: UFLC chromatogram of antidiabetic drugs in plasma samples.

1. **Metformin HCl, 2. Vildagliptin, 3. Gliclazide, 4. Linagliptin, 5. Sitagliptin, 6. Pioglitazone, 7. Glimepiride and 8. Repaglinide.**

4.3.2.1 Ultra Fast Liquid Chromatography Method Optimization

The different combinations of the solvent systems, stationary phases and wavelengths were tried for the optimization purpose. As well, the modification in the flow rate, column temperature, detection wavelength and amount loaded was also carried out. pH of the mobile phase was changed by triethylamine. In addition, the optimization was also achieved by various additives viz. diethyl- and triethyl amines in the mobile phases. After exhaustive experiments, the best UFLC conditions were developed and reported herein.

The effect of acetonitrile amount on the separation of these drugs was done. The content of acetonitrile was varied from 10 to 80 mL. It was observed that the peaks were broad with tailing for 10 to 30 mL of acetonitrile. As a result, the peaks merged into one another at high value of acetonitrile < 40 mL (40-80 mL). Consequently, 40 mL content of acetonitrile was found the best for the excellent base lined separation. The flow rate of the mobile phase was also changed from 0.5 to 1.5 mL min^{-1}. It was seen that at low flow rate of 0.5 mL min^{-1}, the peaks were broaden with high retention times with more than double total run times. Contrarily, at increase flow rates to 1.5 mL min^{-1} some of the peaks were merged into one another. Briefly, the peaks were well resolved at 1.0 mL min^{-1} flow rate. Consequently, this flow rate was chosen.

4.3.2.2 Validation

The validation parameters of UFLC viz. linearity, LOD, LOQ, specificity, precision, accuracy, robustness and ruggedness are discussed below.

4.3.2.2.1 Linearity

The linearity of calibration curves (peak area vs. concentration) for metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were carried out in the concentration ranges of 0.025 to 0.075 mg mL^{-1}.

The plots were linear over these concentration ranges (n = 5) for the reported drugs. The peak areas of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were graphed against their respective concentrations. The linear regression analysis was carried out using the developed curves. The correlation coefficient (r) (n = 5) for metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were 0.9995, 0.9999, 1.0000, 0.9999, 0.9987, 0.9999, 1.0000 and 0.9989. The values of RSD and confidence levels (95% confidence interval) were ranged from 0.059 to 0.52% and 99.18 to 100.37% in the concentration ranges considered.

4.3.2.2.2 Limits of Detection and Quantitation

The LOD and LOQ of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide were 4.98 and 15.09, 2.84 and 8.59, 1.74 and 5.28, 4.11 and 12.44, 8.29 and 25.12, 1.83 and 5.55, 2.97 and 8.99 and 2.74 and 8.29 µg mL^{-1}, correspondingly.

4.3.2.2.3 Specificity

The reported method was pretty specific. The retention times of all the molecules in the standard samples were comparable with those of the plasma samples. There was no affect of the supplemented impurities (in the standards) on the retention times and the peak shapes of these drugs. These results showed good specificity of the reported UFLC method.

4.3.2.2.4 Precision

The precision data was determined at three different concentrations i.e. 0.04, 0.05 and 0.06 mg mL^{-1} of all the reported molecules. Five sets of UFLC runs were carried out for all three concentrations. The values of RSD and confidence levels were ranged from 0.18 to 1.06% and 99.18 to 100.37%.

4.3.2.2.5 Accuracy

The accuracy of the chromatographic method was determined by the different concentrations of the reported molecules. Three concentrations used were 0.001, 0.005 and 0.025 mg mL^{-1}. UFLC experiments were done five times (n = 5). The accuracies were ascertained by interpolation of five replicates peak areas of these drugs. The values of the absolute errors were ranged from 1.62 to 2.00%.

4.3.2.2.6 Robustness

The robustness of the chromatographic method was ascertained by altering slight variations in the UFLC experiments. The diverse experimental settings were solvent system composition, flow rate, temperature and wavelength. The retention time, peak area and shape were analyzed in the developed and slightly varied experimental conditions.

4.3.2.2.7 Ruggedness

The ruggedness of UFLC method was ascertained by altering the experimental conditions such as different operators and several days.

4.4. APPLICATION OF THE DEVELOPED SPE AND UFLC METHODS TO THE REAL WORLD SAMPLES:

The developed and validated SPE and UFLC methods were adopted for the determination of the reported drugs in the human plasma samples. The values for the retention, separation and resolution factors were ranged from 0.073 to 9.15, 1.45 to 4.22 and 2.17 to 18.70, correspondingly. These values were in good agreement with those obtained with the standard drugs. The peak heights were low in comparison to the standard solutions. It was due to the fact that some concentrations of the drugs interacted with plasma proteins. The validation data was close to those obtained with the standard drugs. It was seen that there was no additional peak in the chromatograms, showing fine selectivity of SPE method. Besides, the lack of any extra peak confirmed no new molecule and metabolic product during stay of these drugs in plasma sample. The chromatographic results in terms of retention, separation, resolution factors and symmetry of the eluted peaks were similar to those of the standard samples. These results indicated that the reported SPE and UFLC methods were selective, efficient, rugged and reproducible.

4.5. CONCLUSION:

From the results discussed here, it can be concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of metformin HCl, vildagliptin,

gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide in human plasma. There was no extra peak in the plasma samples, which established the selectivity of SPE method. Also there was no degradation of these drugs in plasma sample. The synchronized separation and identification of these eight drugs are reported first time. The developed SPE and UFLC methods were applied successfully for the monitoring of these drugs in human plasma. Therefore, SPE and UFLC methods may be used for the determination of these drugs in any plasma sample.

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5.1. INTRODUCTION:

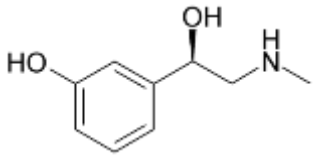
Anti-histamines are drugs which treat allergic rhinitis and other allergies. Anti-histamines can give relief when a person has nasal congestion, sneezing, or hives because of pollen, dust mites, or animal allergy. Typically people take anti-histamines as an inexpensive, generic, over-the-counter drug with few side effects. As an alternative to taking anti-histamine, people who suffer from allergies can instead avoid the substance which irritates them. Anti-histamines are usually for short-term treatment. Chronic allergies increase the risk of health problems which anti-histamines might not treat, including asthma, sinusitis, and lower respiratory tract infection. Although typical people use the word antihistamines to describe drugs for treating allergies, doctors and scientists use the term to describe a class of drug that opposes the activity of histamine receptors in the body. In this sense of the word, anti-histamines are sub-classified according to the histamine receptor that they act upon. The two largest classes of anti-histamines are H1- anti-histamines and H2-antihistamines. Anti-histamines that target the histamine H1-receptor are used to treat allergic reactions in the nose (e.g., itching, runny nose, and sneezing) as well as for insomnia. They are sometimes also used to treat motion sickness or vertigo caused by problems with the inner ear. Anti-histamines that target the histamine H2-receptor are used to treat gastric acid conditions (e.g., peptic ulcers and acid reflux). H1- anti-histamines work by binding to histamine H1 receptors in mast cells, smooth muscle, and endothelium in the body as well as in the tuberomammillary nucleus in the brain; H2- anti-histamines bind to

histamine H2 receptors in the upper gastrointestinal tract, primarily in the stomach.

Histamine receptors exhibit constitutive activity, so anti-histamines can function as either a neutral receptor antagonist or an inverse agonist at histamine receptor [1,2]. Only a few currently marketed H1-anti-histamines are known to function as inverse agonists. Histamine produces increased vascular permeability, rooting fluid to run away from capillary to tissues, which guides to the typical indications of a sensitive to reaction - a runny nose and watery eyes. Histamine also promotes angiogenesis [3,4]. Anti-histamines repress the histamine encouraged wheal answers (swelling) and flare response (vasodilation) by overcrowding the obligatory of histamine to its receptors or reducing histamine receptor activity on nerves, vascular smooth muscle, glandular cells, endothelium, and mast cells [5]. Itching, sneezing, and inflammatory responses are suppressed by anti-histamines that act on H1-receptors [6-8]. In 2014 antihistamines such as desloratadine were found to be effective as adjuvants to standardized treatment of acne due to their anti-inflammatory belongings and their ability to suppress sebum production [9]. There are many anti-histamines drugs but the most important are phenylephrine, cetirizine, loratidine, montelukast and ebastine due to their unique pharmaceutical properties (Fig 5.1). The pharmaceutical properties of these drugs are described in the next section of literature.

Despite of the curative belongings of these medicines, quite a few elevation properties have also been accounted [10]. Usually, these medicines are given in the appearance of the grouping therapy. From time to time, this treatment may

guide a number of stern elevation belongings because of the creation of the extra molecules in the human body. Fascinatingly, the pharmacokinetics and pharmacodynamic of these drugs act in the dissimilar fashion and fluctuate grouping therapies. Consequently, there is a huge necessity to expand precise sample handling and bioanalytical methods. These are described herein.



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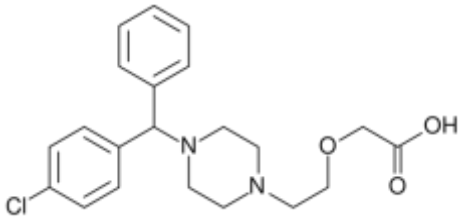
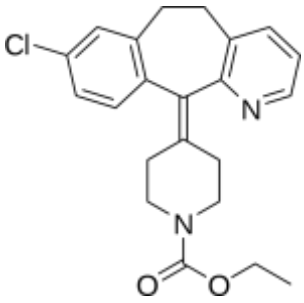
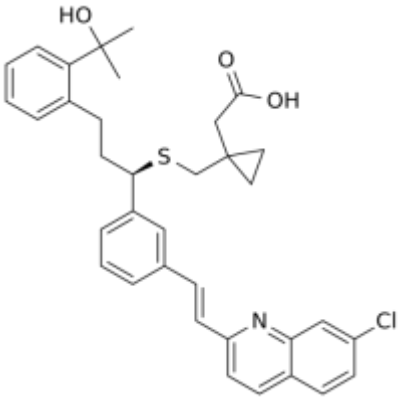


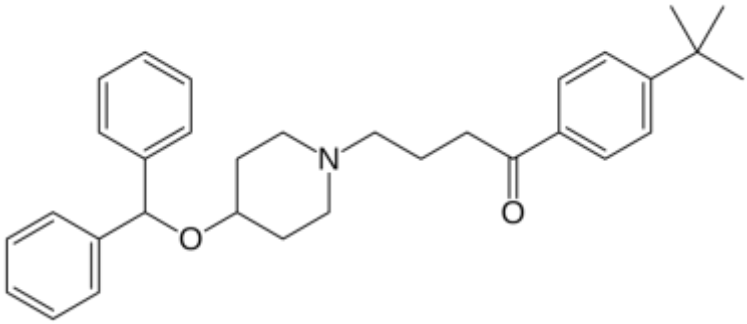
Cetirizine



Loratidine



Montelukast



Ebastine

Figure 5.1: Chemical structures of anti-histamine drugs.

5.2. LITERATURE SURVEY:

The separation and identification includes the chromatographic methodology used to identify antihistamine drugs. These analysis and separation are done by taking the advantage of difference in their properties. The separation and identification of anti-histamine drugs are carried by extraction and separation techniques. The most commonly used techniques are solid phase extraction (SPE) and high performance liquid chromatography (HPLC). A thorough search of literature on the analyses of anti-histamine drugs was carried out through Scifinder, Scopus, and Science direct and peer reviewed Journals. This is discussed in the following sub-sections.

5.2.1 Phenylephrine

A selective α1-adrenergic receptor agonist of the phenethylamine class, Phenylephrine is used mainly as a decongestant, as an agent to open the follower, and to enhance blood pressure [11,12]. For the decongestant pseudoephedrine, phenylephrine is marketed as an alternative, although medical trials show phenylephrine, reserved orally at the recommended amount, to be no more effective than placebo for allergy relief. Phenylephrine can also cause a decrease in heart rate through reflex bradycardia [13,14].

Dewani et al. [15] defined reliable and an easy HPLC-DAD procedure for the phenylephrine analysis HCl in pharmacological combination. The procedure was useful positively on pill dosage form. Effective chromatographic analysis of PAR, PHE and CET was performed by means of a Kinetex-C_{18} stationary phase with gradient elution of the eluent consisted of 10 mM phosphate buffer (pH=3.3)

and ACN. The analytical performance and reliability of the planned HPLC method were mathematically certified with respect to linearity, precision, choices, selectivity, correctness and sturdiness. The authorized HPLC procedure was useful to a pharmacological combination of an advertised training pill in which the analyte was positively quantified with moral recovery magnitudes with no nosey peaks from the excipients. Kumar et al. [16] found an easy, accurate and specific RP-HPLC procedure for the phenylephrine analysis HCl in pharmacological dosage forms. A reversed phase Hypersil phenyl stationary phase with eluent having pH=5.5 consisting of MeOH and buffer (55:45, v/v) was used at 1.0 mL per minute flow rate was and the effluents were watched at 214 nm. The procedure was found to be simple, precise, economical, less time consuming and reproducible for determining phenylephrine in kratom cocktail, Chittrakarn et al. [17] used an easy HPLC technique. The analytical procedure used was an Eclipse XDB-C_8 stationary phase. For analyzing phenylephrine, a Lichrospher CN stationary phase was used. The precision of the procedures were between 0.700 & 7.108% RSD. The correlation coefficient was between 0.9957 & 0.9993.

On the basis of straight removal and HPLC analysis with fluorescence recognition of phenylephrine in pharmacological sachets, Dousa and Gibala [18] defined a fast procedure that include a large paracetamol excess (65 + 1, w/w), ascorbic acid (5 + 1, w/w), and other excipients (sucrose & aspartame) was given and authorized. For ion-pair chromatography, the final optimized chromatographic procedure used an XTerra RP18 stationary phase. The eluent (attuned with H_3PO_4 at pH 2.2; 200 + 800, v/v) composed of a combination of

ACN and buffer (10 mM sodium octane-1-sulfonate, with 0.3 mL per min. flow rate. At 30 °C, the analysis was carried out, and the injection volume was 3 μL. At excn and emissn wavelengths of 275 and 310 nm, fluorescence recognition was performed. The eluent parameters, such as the amount of sodium octane-1-sulfonate as a counter-ion, temperature, and pH of eluent, the organic solvent fraction (ACN) in eluent as an organic modifier were studied. For the analysis of these drugs in pharmacological formulations like capsules and packets, counting the analysis of contaminates and excipients, Marin et al. [19] used a HPLC procedure. The choosiness of the procedure was also tested to be used of phenylephrine. A gradient elution, being solvent A: phosphate buffer 40 mM at pH 6.0 and solvent B: ACN. At t=0, the eluent consisted of 92% A and 8% B and it changed with a linear gradient during 8 min to 75% A and 25% B was final chromatographic circumstances. At t=15 min, it returns to the initial circumstances (92% A and 8% B), at min 8, it distorted to 30% A and 70% B for 5 min and during 1 min residual at this concerto until t=20 min. Besides, to build up and validate an isocratic HPLC procedure for the Phenylephrine analysis in capsules as pharmacological formulation, the same group [20] used a new polar reverse phase column. The eluent was 20 mM phosphate buffer at pH 7.0/ACN 80:20 (v/v) at 1 mL per min flow rate. Final optimized chromatographic circumstances in work a Supelco Discovery HS PEG stationary phase (polyethylene glycol).

Hudecová et al. [21] defined a fast HPLC procedure for the Phenylephrine analysis in eye drops. The procedure uses a modified C_{18} column optimized for

the analysis of basic compounds and a MeOH/1.5 mM phosphoric acid (60/40 v/v, pH 3.02) eluent. The flow rate was set to 2 mL per min., sample volume 20 μL. Prior to analysis, the eye drops are diluted with H_2O in a ratio of 1:50. The elaborated HPLC procedure and the chromatographic system were authorized according to the procedure for the validation of chromatographic systems and procedures. Gumbhir and Mason [22] found an HPLC procedure for the phenylephrine analysis and its conjugates in plasma of human. The examination was done out on a 15 cm ODS stationary phase by means of ion pair reversed phase chromatography. An electrochemical sensor operated at + 1.15 V vs. Ag/AgCl was engaged for recognition. In the range 1.0-50.0 ng per ml for phenylephrine, the standard curves were linear. Acceptable precision and accuracy were obtained during inter- and intra- batch analyses. Yamaguchi et al. [23] found a HPLC with fluorescence recognition for the total phenylephrine analysis (free plus conjugated) in serum of human. Low limit of quantitation for phenylephrine in serum of human was 5 ng per ml. After oral administration of phenylephrine HCl, the procedure was useful to the Phenylephrine analysis in serum of human.

5.2.2 Cetirizine

A second generation anti-histamine and a main metabolite of hydroxyzine, and a racemic selective H1 receptor antagonist, Cetirizine is used in the management of hay fever, angioedema, allergies, and urticaria. Like cetirizine, second generation anti-histamines are less capable to cross the blood brain fence and, consequently, have diminished effects on the central nervous system compared to 1st generation drugs: for instance they are less likely to induce

drowsiness or to interfere with memory formation. Cetirizine is also commonly prescribed to treat acute and (in particular cases) chronic urticaria, more efficiently than any other second-generation antihistamine [24,25]. Cetirizine's primary indication is for hay fever and other allergies. Because the symptoms of itching and redness in these circumstances are caused by histamine acting on the H1 receptor, blocking those receptors temporarily relieves those symptoms [26,27].

Flórez Borges et al. [28] defined an easy, new and accurate constancy-indicating reverse phase HPLC for cetirizine diHCl. On Eclipse XDB® C_8 stationary phase, RP-HPLC analysis was carried out with an eluent consisted of a combination of 0.2 M potassium hydrogen phosphate pH 7.00 and ACN (65:35, v/v) at 1 mL per min flow rate. At 230 nm by means of diode array sensor, recognition was performed. The procedure results in excellent analysis between the drug substance and its stress-induced degradation products. The proposed constancy-indicating procedure given in the early phase of drug development proved to be sensitive simple, precise accurate, and reproducible. Souri et al. [29] found a HPLC procedure and used to learning cetirizine degradation of dil. HCl in acidic and oxidative circumstances. On a Symmetry C_{18} stationary phase, the analysis was carried out and a combination of 50 mM KH_2PO_4 and ACN (60:40 v/v, pH 3.5) was used as the eluent. For the cetirizine analysis in plasma of human by means of nebivolol as the internal standard, Dharuman et al. [30] defined a stationary phase switching HPLC procedure with estimable sensitivity and accuracy. By liquid-liquid extraction in methylene chloride and a combination of

diethylether (80:20, v/v). The drug was analysed to an analytical Kromasil C_8 micro bore stationary switching device. Eluent consisted ACN-TEA (0.5%) in phosphate buffer (pH 3.5; 20mM) (55:45, v/v). For cetirizine, the procedure was linear from 2 to 450 ng per ml and 7-300 ng per ml.

Ren et al. [31] defined a fast, selective and sensitive HPLC-MS/ MS procedure for cetirizine quantification dil. HCl in plasma of human by means of internal standard, mosapride citrate. On a reverse phase C_{18} stationary phase, the analyte was separated following liquid-liquid extraction by means of an eluent consisting of MeOH and aqueous CH_3COONH_4 solution (10 mM) (60:40, v/v). The lower limit of quantification (LLOQ) was 0.5 ng per ml with a relative standard deviation of < 15% (all the concentration data in this study related to the salt (cetirizine dil.HCl)). For concentrations over the standard curve range, acceptable accuracy and precision were obtained. Hadad et al. [32] defined a RP-HPLC procedure for the purpose of conducting constancy studies of the cetrazine. The stationary phase used was reversed-phase HS C_{18}. With UV recognition at 214 nm, based on peak area, quantitation was performed. The proposed procedure was authorized and positively useful for the analysis of pharmacological formulation and laboratory-prepared combinations. Bhatia et al. [33] used a fast, precise, accurate, specific and sensitive reverse phase liquid chromatographic procedure for the cetirizine HCl analysis in their pill formulation. The chromatographic procedures were standardized by means of a HIQ SIL-C_{18} stationary phase with UV recognition at 229 nm and eluent consisting of MeOH-ACN-H_2O (40:40:20, v/v). The proposed procedure was positively useful to the

cetirizine HCl analysis in pills, with high percentage of recovery, good accuracy and acceptable precision.

Karakuş et al. [34] defined SPE and HPLC procedures for analysis of cetrazine in dosage forms. On a Zorbax C_8 stationary phase, the chromatographic PSE, FEX and CET analysis was performed. The optimized eluent was comprised of TEA solution (0.5%, pH 4.5) − MeOH-ACN (50:20:30, v/v). Within the concentration ranges 30 − 240 and 1.25 − 10 μg per ml, the proposed procedure provided linear responses. Correlation coefficients (r) of the regression equations were > 0.999. Ma et al. [35] described a highly selective, sensitive, simple and liquid chromatography/tandem mass spectrometry (LC/MS/MS) procedure to analyse simultaneously cetirizine concentrations in plasma of human. On a C_8 stationary phase, the drug was separated and analyzed by LC/MS/MS by means of positive electrospray ionisation (ESI). Over the concentration range of 1.0−400 ng per ml, the procedure had a chromatographic run 12.0 min time and a linear calibration curve. 1.0 ng per ml was the lower limit of quantification of the drug. With electrospray ionization (HPLC-ESI-ion trap mass spectrometry) procedure for cetirizine analysis in plasma of human, Tan et al. [36] used a liquid chromatography-ion trap mass spectrometry. On a Hypurity C_{18} stationary phase, Chromatographic analysis was performed. The eluent was consisted of 65% MeOH and 35% H2O (contained 0.1% HCOOH, 10 mM $HCOONH_4$), which was run at 40 °C with 0.2 mL per min flow-rate. 5 ng per ml was the limit of recognition. For cetirizine analysis in plasma of human, this simplified analytical procedure was specific, sensitive and accurate enough.

Arayne et al. [37] found an easy, reliable, sensitive, and fast HPLC procedure for the cetirizine HCl analysis by means of hyoscine butyl bromide as an internal standard has been given. With UV/visible sensor and a CBM-102 Bus Module integrator, the chromatographic system consisted of Shimadzu LC-10 AT VP pump, SPD-10 AV VP. Analysis was performed on the U Bondapak 125 A C_{18} 10 μm stationary phase at room temperature. The samples were introduced through an injector valve with a 10 μm sample loop. ACN-H_2O (1:1, v/v) was used as eluent, with 2 mL per min flow rate. pH was adjusted to 2.9 with phosphoric acid. At 205 nm, UV recognition was performed. With the declared content, the results obtained showed a good agreement. Recovery values for cetirizine HCl were 99.19-100.82%. For the quantitative cetirizine HCl analysis, the proposed procedure was reliable fast, precise, selective and may be used, in presence of hyoscine butyl bromide as internal standard. The procedure was valid was for the analysis in raw materials, bulk drug and formulations. The limit of quantification was 5-30 nano grams, while the limit of recognition was 0.4 nano grams. Kim et al. [38] found an improved stationary phase switching high performance liquid chromatographic (HPLC) procedure for cetirizine analysis in plasma of human. By liquid-liquid extraction by means of methylene chloride, plasma samples were prepared. The samples extracted were initially injected into a clean-up Capcell Pak MF C_8 stationary phase and cetirizine peaks and internal standard were separated to an analytical C_{18} micro-stationary phase via stationary phase switching device. This analysis showed highly selective and sensitive results. Also, it was positively useful to evaluate cetirizine pharmacokinetics in

human volunteers after single oral administration. Jaber et al. [39] gave an HPLC procedure for the cetirizine HCl analysisas well as its related impurities in commercial oral solution and pill formulations. With a Hypersil BDS C_{18}, stationary phase and a sensor set at 230 nm in conjunction, the chromatographic system used was prepared and 0.05 M dihydrogen phosphate : ACN : MeOH : tetrahydrofuran (12 : 5 : 2 :1, v/v) at a pH of 5.5 was used an eluent at 1 mL per min flow rate. Within the target concentration ranges studied, the calibration curves were linear. The procedure shown to be precise, robust, accurate, specific, constancy indicating, and could be used as an alternative to the European pharmacopoeial procedure set for the medicine and its related impurities.

5.2.3 Loratidine

Loratadine is sold under the brand name Claritin and a medication used to treat allergies. Loratadine was discovered in 1981 and came to market in 1993. This includes allergic rhinitis (hay fever) and hives. It is also available in combination with pseudoephedrine, a decongestant, known as loratadine/pseudoephedrine. Loratadine is indicated for the symptomatic relief of allergy such as hay fever (allergic rhinitis), urticaria (hives), chronic idiopathic urticaria, and other skin allergies. Loratadine is effectual for together nasal and eye indications, sneezing, liquid nose, itchy or burning eyes for allergic rhinitis (hay fever). Similarly to cetirizine, loratadine attenuates the itching associated with Kimura's disease. It is marketed for its less sedating properties. The common side effects comprise dry mouth, sleepiness, and headache. Serious side effects are rare and include allergic reactions, seizures, and liver problems. Use during

pregnancy appears to be safe but has not been well studied. It is not recommended in children less than two years old. It is in the second-generation antihistamine family of medication [40,41].

Farid and Abdelaleem [42] defined an accurate sensitive, and HPTLC procedure for loratidine analysis. Pre-coated with silica gel 60 F_{254}, the proposed chromatographic procedure has been given at 254 nm for PAR, 4-AP and LOR by means of HPTLC aluminum plates by means of CH_3COCH_3-C_6H_{14}- NH_3 (4 : 5 : 0.1, v/v) as an eluent pursuing by densitometric extent, while PSH was scanned at 208 nm. To determine the excellence presentation of the given chromatographic procedure, system fitness testing parameters were calculated. With respect to USP rules concerning correctness, accuracy and specificity, the procedure was authorized. The procedure was positively useful for the drug analysis in the pills. The result obtained by a found HPLC procedure was compared with the results obtained by the proposed procedure, and there was no meaning difference between both procedures regarding precision and accuracy.

5.2.4 Montelukast

Montelukast, a leukotriene receptor antagonist used to mitigated indications of recurrent allergies and for the maintenance treatment of asthma comes as a pill, a chewable pill, flash pill and granules to take by mouth. Montelukast, blocking the act of leukotriene D4 (and secondary ligands LTC4 and LTE4) on the cysteinyl leukotriene receptor CysLT1 in the lungs and bronchial tubes by binding to it, is a CysLT1 antagonist. This decreases the broncho-constriction or else rooted by the leukotriene and consequences in less

inflammation. Since its mechanism of action, it is not helpful in the management of acute asthma assaults. Montelukast is used for a number of circumstances including asthma, exercise induced bronchospasm, allergic rhinitis, primary dysmenorrhoea (i.e. dysmenorrhoea not associated with known causes; see dysmenorrhea causes), and urticaria. It is mostly utilized as a complementary therapy in adults in accumulation to inhaled corticosteroids, if they alone do not bring the desired effect. It is also used to prevent allergic reactions and asthma flare-ups during the administration of intravenous immunoglobulin.

The common side effects include diarrhea, nausea, vomiting, mild rashes, asymptomatic elevations in liver enzymes, and fever. Uncommon side effects include fatigue and malaise, behavioral changes, paresthesias and seizures, muscle cramps, and nose bleeds. Rare but sometimes serious side effects include severe behavioral changes (including suicidal thoughts), angioedema, erythema multiforme, and liver problems [43-46].

Ranjan et al. [47] found a liquid-liquid extraction based reverse phase liquid chromatography procedure for the montelukast estimation in Plasma of rabbit. The chromatographic analysis was done by means of Phenomenex Luna C_{18} stationary phase with eluent consisted of CH_3COONH_4 buffer (20 Mm), pH 5.5 and ACN in 20 : 80, v/v ratio. The analyte was watched with UV sensor at 345 nm. The given procedure was authorized with respect to specificity, linearity, correctness, exactitude, and steadiness. The peak area ratio of to that of internal standard was used for the samples quantification. In the amount range of 20-2000 ng per ml, calibration curves were linear. The LOD and LLOQ of present

procedure were found out to be 10 ng per ml and 20 ng per ml, correspondingly. Extraction recoveries of drug from Plasma of rabbit were >66.47%. An easy, alternative, reproducible and sensitive HPLC-UV procedure was given that can be used in preclinical preparations. Pankhaniya et al. [48] used an easy, specific, constancy-indicating, accurate, and reversed-phase HPLC procedure for the montelukast analysis by means of a Lichrospher(®) 100, RP-18e stationary phase and a eluent consisted of MeOH:0.1% o-phosphoric acid (90:10 v/v), pH 6.8. For montelukast HCl in the range of 2-10 μg per ml, linearity was established. The % recovery of montelukast was found to be 99.09. For quantitative montelukast analysis in bulk drugs and formulations, this procedure can be positively employed. Revathi et al. [49] used high performance liquid chromatography (HPLC) procedure for single component or multi-component montelukast analysis sodium with other drugs. The analysis was performed on an inertsil stationary phase in isocratic mode with the eluent composed of a combination of MeOH and Na_3PO_4 buffer (75:25 v/v, pH 6.5 adjusted with H_3PO_4). The eluent was pumped at 1 mL per min flow rate and eluents were watched at 230 nm. The proposed HPLC procedure was authorized with respect to accuracy, linearity, repeatability, robustness, specificity, and ruggedness as per International Conference on Harmonisation guidelines Q2(R1), November 2005 (Validation of Analytical Procedures: Text and Procedureology). It was concluded that the given procedure was fit for routine drug analysis due to its less analysis time.

Challa et al. [50] found an easy, responsive, and exact LC-ESI/MS/MS procedure for Montelukast quantification in plasma of human by means of

Montelukast-d(6) as an internal standard (IS). Chromatographic analysis was done on YMC-pack pro C_{18}. The eluent was 10 mM ammonium formate (pH 4.0):ACN (20:80 v/v), at 0.8 mL per min flow-rate. Both analytes were originated to be stable throughout 3 freeze-thawing series, bench upper, and auto-sampler constancy studies. This procedure was utilized positively for plasma samples analysis of following oral administration of the drug in 31 healthy Indian male human volunteers under fasting circumstances. Singh et al. [51] used an accurate HPLC procedure for montelukast sodium estimation in pill dosage form and in bulk. The analysis was performed by by means of octadecylsilane stationary phase C_{18} and CAN : 1 mM CH_3COONa adjusted to pH 6.3 with CH_3COOH in proportion of 90:10 v/v as eluent, at 1.5 mL per min flow rate. At 285 nm, recognition was carried out. Montelukast sodium retention time was found to be 3.4 min. The limit of recognition was found 1.31 µg/ml and limit of quantification 3.97 µg/ml. By evaluating various validation parameters like linearity (1-100 µg/ml), precision, accuracy and specificity according to ICH guidelines, the reliability and accuracy of the planned procedure was ascertained. The proposed procedure provided an accurate and precise quality control tool for routine montelukast analysis sodium in bulk and in pill dosage form. Patil et al. [52] used an accurate, specific and precise assay level gradient reverse-phase high-performance liquid chromatographic procedure for montelukast analysis sodium in pill dosage form. An inertsil ODS C_{18}, 5 µm stationary phase having 250 x 4.6 mm I.D. in gradient mode, with eluent A, containing 0.025 M sodium phosphate buffer: MeOH (85:15) and eluent B, containing ACN : MeOH (85:15) was used at

different time intervals. The effluent was watched at 218 nm and at 1.5 mL per min. flow rate. The linearity was in the range of 0.25-0.75 mg per ml with correlation coefficients of 0.9999 for montelukast sodium.

Radhakrishna et al. [53] found a HPLC procedure for the montelukast analysis in pharmacological formulations. HPLC analysis was performed with a Symmetry C_{18} stationary phase and Na_3PO_4 buffer (pH adjusted to 3.7): ACN (20:80, v/v) as eluent, at 1.0 mL per min flow rate. At 225 nm, UV recognition was performed. The procedure was fast, simple, selective and constancy indicating for the montelukast analysis. 5-Methyl 2-nitrophenol was used as internal standard for the purpose of quantification of the drug in HPLC. Al-Rawithi et al. [54] defined an HPLC procedure for montelukast sodium analysis in plasma of human. The drug and internal standard, quinine bisulfate, were measured by HPLC after an easy extraction of the plasma. At 40 °C and a fluorescence sensor with the excitation and emission wavelengths set at 350 and 400 nm, the chromatographic system consisted of a refrigerated autosampler, a single pump, a C_8 4-μm particle size radial compression cartridge. By adding 200 ml of 0.025 M sodium acetate, pH adjusted to 4.0 with CH_3COOH, to 800 ml of ACN, with 50 μL TEA, The eluent which was delivered at 1.0 mL per min., was prepared. With fluorescence recognition for analysis of montelukast sodium in plasma of human, Ochiai et al. [55] defined a stationary phase-switching HPLC. Onto the HPLC system consisting of a pre-stationary phase (Capcell pak MF) and an analytical stationary phase (Capcell pak C_{18}) which were connected with a six-port switching valve, a plasma sample was injected directly. The stationary phase

eluate was watched with a fluorescence sensor (excitation at 350 nm; emission at 400 nm). The calibration curve was linear in a concentration range of 1-500 ng per ml in plasma of human. The intra-day coefficients of variation of all concentrations within the range were < 9.2%, and the intra-day accuracy values were between 97.2 & 114.6%. This procedure was used to measure drug concentration in plasma the following oral administration of the drug in humans.

5.2.5 Ebastine

Ebastine is a H1 antihistamine with low potential for caby means of drowsiness. It does not penetrate the blood brain barrier to a significant amount and, thus, combines an effective block of the H1 receptor in peripheral tissue with a low incidence of central side effects, i.e. seldom caby means of sedation or drowsiness. A second-generation H_1 receptor, Ebastine is antagonist that is designated mostly for allergic rhinitis and chronic idiopathic urticaria. It is available in 10 and 20 mg pills and as fast dissolving pills, as well as in pediatric syrup. It has a recommended flexible daily dose of 10 or 20 mg, depending on disease severity. Ebastine has shown overall safety and tolerability profile with no cognitive/psychomotor impairment and no sedation worse than placebo, and cardiac safety, that is, no QT prolongation. The incidence of most commonly found adverse events was comparable between the ebastine and placebo groups, which confirms that ebastine has a favourable safety profile. While experiments in pregnant animals showed no risk for the unborn, no such data are available in humans. It is not known whether ebastine passes into the breast milk [56-60].

For purity testing of ebastine and its pharmacological formulations, Schmidt et al. [61] defined a constancy-indicating UHPLC procedure. On an Acquity UPLC BEH C_{18}, stationary phase successful chromatographic analysis of the API from impurities was performed. 10 mM acetate buffer pH 6.2 and a combination of ACN/2-propanol (1:1) as the eluent was used. The procedure was fully authorized for linearity, specificity, precision, accuracy and robustness in compliance to the ICH guideline Q2 (R1). By varying the six parameters: gradient time, ternary composition of the eluent, flow rate, temperature, and start and end concentration of the gradient at 3 levels (+1, 0, -1), the robustness of the given procedure was studied. Haggag and Belal [62] defined a high-performance liquid chromatography-diode array recognition (HPLC−DAD) procedure for ebastinethe analysis. With gradient elution of the eluent consisted of 0.05M F and CAN, effective chromatographic analysis of EBS, PSD and PHR was performed by means of a Zorbax SB-C_8 (4.6 x 250 mm, 5 μm) stationary phase. Started with 20% (by volume) ACN, the gradient elution, ramped up linearly to 90% in 5 min, then kept constant until the end of the run at 1 mL per min flow rate. At 254 (for EBS and PSD) and 274 nm (for PHR), the multiple wavelength sensor was set and analyte quantification was based on measuring the peak areas. The analytical performance and reliability of the proposed HPLC method were statistically authorized with respect to precision, linearity, accuracy, ranges, selectivity, robustness and quantification and recognition limits. The authorized HPLC procedure was useful to the drug analysis in laboratory-made pills. Prabu et al. [63] found an easy, precise and fast RP-HPLC procedure for ebastinethe analysis

in pharmacological formulations. The procedure was carried out on a Phenomenex RP-C_{18} stationary phase by means of a combination of MeOH and H2O (90:10) and recognition was done at 262 nm. The linearity range was 5-100 mug/ml. The inter-day and intra-day precision were in the range of 0.22% to 0.49% & 0.24% to 0.73%, correspondingly.

Matsuda et al. [64] defined an easy and sensitive HPLC procedure for ebastinethe analysis and its carebastine, two oxidized metabolites, and hydroxyebastine in plasma of human. On an HPLC system with ultraviolet recognition at 254 nm after a pretreatment of plasma sample by solid-phase extraction, ebastine and its metabolites were analyzed. On a cyano stationary phase at 40 ºC, chromatography was performed with the eluent of ACN-MeOH-0.012 M CH_3COONH_4 buffer (20:30:48, v/v) at 1.2 mL per min flow rate. Over the concentration range of 3-1000 ng per ml, accurate analysis were possible for 3 compounds by means of 1 ml plasma samples. The inter- and intra- day assay accuracy of this procedure was within 100±15% of nominal values and the precision did not exceed 12.4% of relative standard deviation. For ebastine and its metabolites in plasma of human, the lower limits of quantitation were 3 ng per ml. After oral administration ofebastine, this procedure was satisfactorily useful to ebastinethe analysis and its two oxidized metabolites in plasma of human. Rana et al. [65] defined a fast and sensitive RP-HPLC procedure with UV recognition (244 nm) for routine ebastine analysis in a pharmacological formulation. Chromatography was performed with eluent containing a combination of MeOH:ACN:CH_3COONH_4 (80:10:10, % v/v), pH of eluent was adjusted 5.5 by

means of glacial CH_3COOH at 1.2 mL per min flow rate. The procedure was authorized for accuracy, linearity, robustness and intermediate precision. Over the concentration range of 0.01-0.06 mg per ml, the linearity was established. The correlation coefficients (r^2) for ebastine were 0.9989. Statistical analysis of the data presented that the procedure was accurate, precise, reproducible and selective for ebastinethe analysis drug. The procedure was positively employed for ebastinethe analysis in commercially available pill dosage form.

5.3. RESULTS AND DISCUSSION:

The consequences and argument is divided into 2 parts viz. UFLC & solid phase extraction which are discussed in the following sub-sections.

5.3.1 Solid Phase Extraction

To separate these drugs from the plasma samples, solid phase extraction procedure was utilized. The % recoveries of phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine were determined by doing the blank experiments. The intended percentage recoveries of phenylephrine HCl, cetirizine HCl, loratidine HCl, montelukast sodium and ebastine in laboratory synthesized samples in H_2O were 80, 78, 85, 94 and 71%, correspondingly. These values in plasma were 10, 12, 15, 06 and 29%, correspondingly (Table 5.1). In the plasma samples, low values were due to the required of these medications with proteins of plasma.

By varying the different SPE circumstances viz. concentrations and pHs of phosphate buffer and the flow rates of plasma samples, phosphate buffer and eluting solvents, SPE optimization was done. The extra eluting solvents viz.

CH_2Cl_2, EtOH, acetone and $CH_3COOC_2H_5$ were also used. As well, some acid additives such CH_3COOH, CCl_3COOH and CF_3COOH were also used. Thus, the best eluting solvent was MeOH. The max. % recoveries of these drugs were obtained by phosphate buffer (20.0 mM, pH 7.0), correspondingly and independently, at 0.1 mL per min flow rate. The values of correlation coefficient (R), RSD, and confidence level for these medicines were ranged from 1.2 to 1.5, 0.9994 to 0.9997 and 99.0 to 99.1, correspondingly (Table 5.2).

Table 5.1. The percentage recoveries of anti-histamine drugs in the laboratory synthesized samples in water and human plasma samples using solid phase extraction.

S.No.	Name of drugs	% Recovery in water	% Recovery in human plasma
1.	Phenylephrine HCl	80	10
2.	Cetirizine HCl	78	12
3.	Loratidine HCl	85	15
4.	Montelukast sodium	94	06
5.	Ebastine	71	29

Table 5.2. Validation data of SPE method for antidiabetic drugs.

S.No.	Drugs	%RSD	Correlation Coefficient (r)	Confidence Level (%)
1.	Phenylephrine HCl	1.5	0.9996	99.0
2.	Cetirizine HCl	1.6	0.9997	99.0
3.	Loratidine HCl	1.3	0.9995	99.1
4.	Montelukast sodium	1.2	0.9994	99.1
5.	Ebastine	1.4	0.9996	99.0

5.3.1.1 Optimization

As usual solid phase extraction is the best tool for extraction of various molecules. The same method was applied for extraction of cardio anti-histamine drugs in human plasma. The percentage recoveries of anti-histamine drugs from standard samples ranged between 71 to 94 %, respectively. These values were from 06 to 29% in plasma samples. These results indicated that solid phase extraction was an optimum method for the extraction of cardio vascular drugs. The recoveries were less in plasma samples as compared to standard samples because in case of plasma samples some amount of anti-histamine drugs interacted with the protein molecules and cannot be extracted by SPE. It is also interesting to observe that in HPLC chromatograms no extra peaks were found, which shows the selectivity of the solid phase extraction method. SPE process was also optimized by pH of the plasma, flow rate of plasma and flow rate of

eluting solvent. Besides, various eluting solvents *viz*. methanol, dichloromethane, ethanol, acetone and ethyl acetate were used for the optimization of the solid phase extraction method. An extensive experimentation was carried out for optimizing SPE conditions and the experimental procedure is reported in the following sections.

5.3.1.1.1 Effect of pH of Plasma

The percentage recoveries of the analytes change according to the pH of the plasma. In solid phase extraction, the purification of the analyte from the plasma occurs on the basis of adsorption on the silica gel of the C_{18} cartridge. This process of adsorption is mainly governed by the adjustment of plasma pH. The pH range from 1.0 to 10.0 was used in this extraction process, to find out the variation in percentage recoveries with the change in the pH of the plasma. The Figure 5.2 shows the effect of various pH ranges on the percentage recoveries of anti-histamine drugs. It is clear from the figure that at lower pH values the percentage recoveries are poor and increased up to 7.0 pH. Further increase in pH also could not increase the extraction percentage recoveries in the standard solutions. The maximum percentage recoveries of the drugs at pH 7.0 were in the range of 71-94%. The reason of the various values at pH 7.0 I due to different ionic strength of thee drugs.

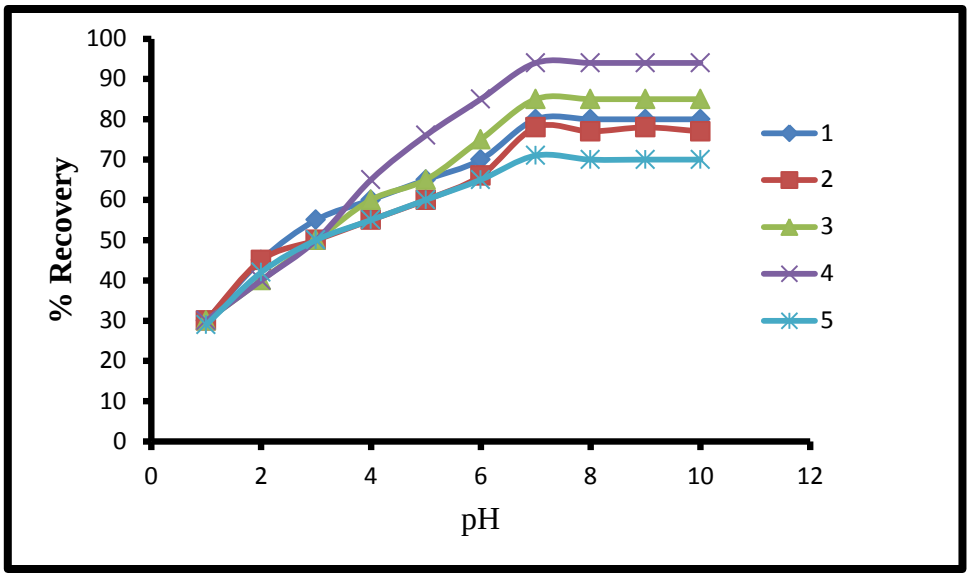


Figure 5.2: The effect of different pH of plasma on the percentage recoveries of anti-histamine drugs.

1. **Phenylephrine HCl, 2. Cetirizine HCL, 3. Loratidine HCl, 4. Montelukast sodium and 5. Ebastine.**

5.3.1.1.2 Effect of Flow Rate of Plasma

The flow rate is one of the most important optimizing factors in solid phase extraction. Generally, high percentage recoveries of analytes are obtained at slow flow rate of plasma whereas fast flow rate leads to the poor percentage recoveries of the analytes. Because of this, efforts were made towards the proper optimization of the solid phase extraction for maximum recoveries of the drugs. For this purpose various flow rates such as 0.025, 0.05, 0.075, 0.10, 0.125 and 0.15 mL/min were tried in the extraction process. The results of the experiments are shown in Figure 5.3 and this figure clearly shows that percentage recoveries of the anti-diabetic drugs ranged from 71 to 94 at different flow rates. Of course, the maximum percentage recoveries were at 0.025 mL/min flow rate but this flow rate was too low to use experimentally. Keeping all the facts, into consideration, 0.10

ml/min flow rate was considered worthwhile and the maximum percentage recoveries at this flow rate were 71 to 94 percent. Therefore this flow rate was used throughout this study.

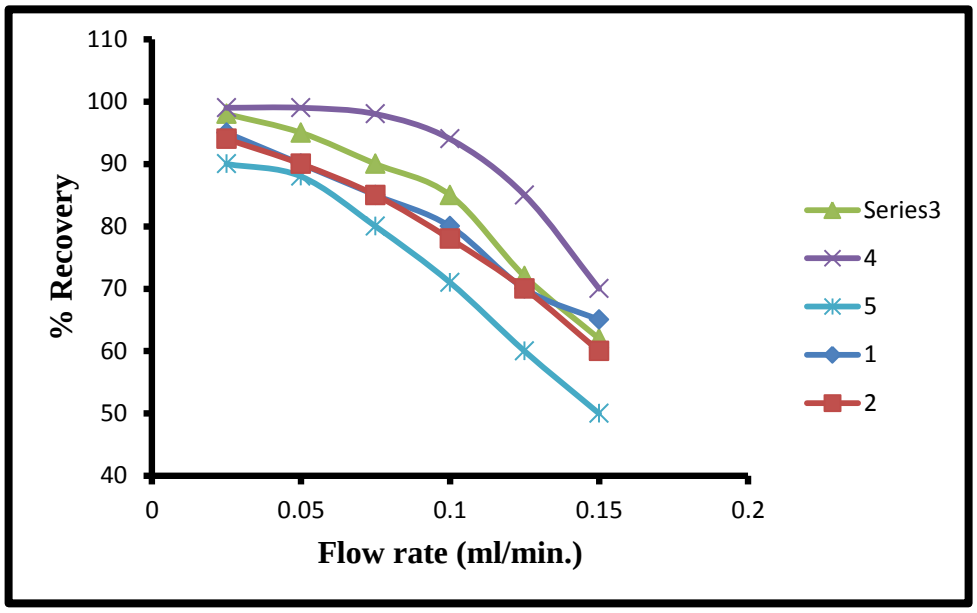


Figure 5.3: The effect of different flow rates of plasma on the percentage recoveries of anti-histamine drugs.

1. **Phenylephrine HCl, 2. Cetirizine HCL, 3. Loratidine HCl, 4. Montelukast sodium and 5. Ebastine.**

5.3.1.1.3 Effect of Other Solvents

The selection of suitable eluting solvent also governs the optimization of the solid phase extraction. So, in order to investigate the best eluting solvent for the elution of essential amino acids through the C_{18} cartridge various solvents were tried. Methanol, dichloromethane, ethanol, ethanol, acetone and ethyl acetate were used as the eluting medium to obtain the maximum recoveries of the anti-diabetic drugs from the C_{18} cartridge. The percentage recoveries of the drugs

obtained by the use of these five solvents are presented in the Figure 5.4. It is very clear from this figure that the order of percentage recoveries of the analytes is methanol > dichloromethane > ethanol > acetone > ethyl acaetae. A critical evaluation of this set of experiment indicated that the maximum and minimum percentage recoveries of the drugs were obtained by using methanol and ether, respectively. Therefore, it was concluded that methanol was the best eluting solvent for the maximum recoveries of the drugs through the C_{18} cartridge. Different polarities and dielectric constant values of these solvents are responsible for their different behavior. Among these solvents methanol is more proficient in the process of desorption of the analytes through the C_{18} cartridge as it has good dielectric constant value and polarity than that of others. Besides methanol, dichloromethane also has considerable values of dielectric constant and polarity and, thus, also gives good percentage recoveries of the drugs. But the use of methylene dichloride was avoided in the experiments because of its volatile nature, which is not safe for the experimental operator. And for the remaining solvents the values of dielectric constants and polarities were not significant to cause the bond dissociation between the molecules of the reported drugs and C_{18} material of solid phase cartridge. Due to which they gave low values of percentage recoveries of the reported drugs. Therefore, methanol was proved to be the best eluting solvent and it was used throughout the experiments.

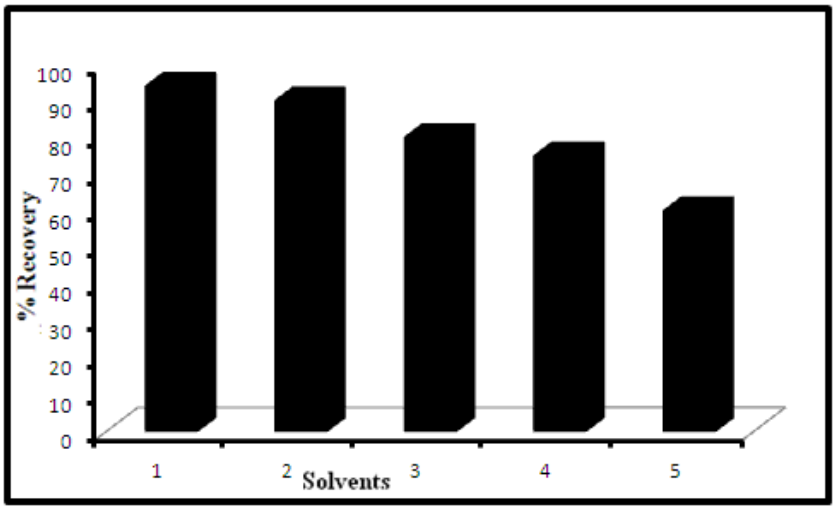


Figure 5.4: The effect of different solvents on the percentage recoveries of anti-histamine drugs.

1. **Phenylephrine HCl, 2. Cetirizine HCL, 3. Loratidine HCl, 4. Montelukast sodium and 5. Ebastine.**

5.3.2 Ultra Fast Liquid Chromatography

The ultra fast liquid chromatographic parameters viz. retention (k), separation (α), and resolution (Rs) factors were determined for phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine. The values of these UFLC parameters are given in Table 5.3. The values of the retention, separation and resolution factors were ranged from ranged from 2.00 to 11.00, 1.15 to 2.31 and 1.00 to 6.07, correspondingly. The UFLC chromatograms of these drugs in standard and plasma samples are reported in Figure 5.5 and 5.6, respectively. It is apparent from this figure that the described drugs were base line separated. The identification of the separated drugs was determined by running and comparing the retention times of the individual phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine. It was observed that there

was no additional peak in the plasma samples, which established the selectivity of the SPE method.

Table 5.3: Chromatographic parameters i.e. capacity factors (k), separation factors (α), resolution factors (Rs) of anti-histamine drugs.

S. No.	Anti-histamines	k	α	R_s
1.	Phenylephrine-HCl	2.00	-	-
2.	Cetirizine-HCl	2.60	1.30	1.00
3.	Loratidine	6.00	2.31	6.07
4.	Montelukast sodium	9.60	1.60	4.95
5.	Ebastine	11.00	1.15	1.67

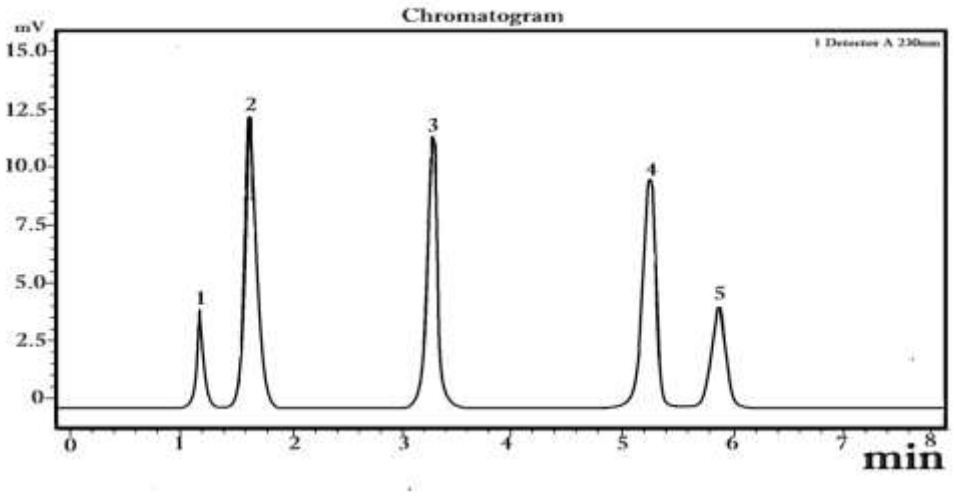


Figure 5.5: UFLC chromatogram of anti-histamine drugs in standard solutions.

1. **Phenylephrine HCl, 2. Cetirizine HCl, 3. Loratidine HCl, 4. Montelukast Sodium and 5. Ebastine.**

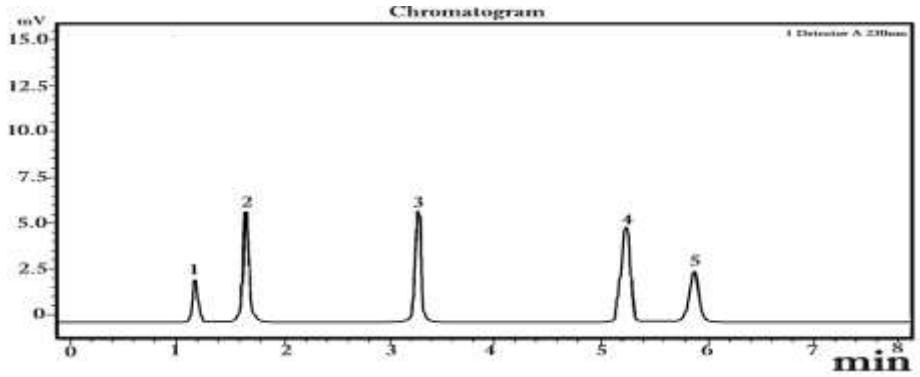


Figure 5.6: UFLC chromatogram of anti-histamine drugs in plasma samples.

1. **Phenylephrine HCl, 2. Cetirizine HCl, 3. Loratidine HCl, 4. Montelukast Sodium and 5. Ebastine.**

5.3.2.1 Ultra Fast Liquid Chromatography Method Optimization

The different combinations of the solvent systems, stationary phases and wavelengths were tried for the optimization purpose. As well, the modification in the flow rate, column temperature, detection wavelength and amount loaded was also carried out. pH of the mobile phase was changed by triethylamine. In addition, the optimization was also achieved by various additives viz. diethyl- and triethyl amines in the mobile phases. After exhaustive experiments, the best UFLC conditions were developed and reported herein.

The effect of acetonitrile and ethanol amounts on the separation of these drugs was done. The contents of ethanol and acetonitrile were varied from 10 to 80 mL. It was observed that the peaks were broad with tailing for 10 to 30 mL of acetonitrile and ethanol. As a result, the peaks merged into one another at high value of ethanol and acetonitrile < 40 mL (40-80 mL). Consequently, 25 and 30 mL contents of acetonitrile and ethanol were found the best for the excellent base

lined separation. The flow rate of the mobile phase was also changed from 0.5 to 1.5 mL min^{-1}. It was seen that at low flow rate of 0.5 mL min^{-1}, the peaks were broaden with high retention times with more than double total run times. Contrarily, at increase flow rates to 1.5 mL min^{-1} some of the peaks were merged into one another. Briefly, the peaks were well resolved at 1.0 mL min^{-1} flow rate. Consequently, this flow rate was chosen.

5.3.2.2 Validation

The validation parameters of UFLC viz. linearity, LOD, LOQ, specificity, precision, accuracy, robustness and ruggedness are discussed below.

5.3.2.2.1 Linearity

The linearity of calibration curves (peak area *vs.* concentration) for Phenylephrine HCl, Cetirizine HCl, Loratadine HCl, Montelukast sodium and Ebastine were carried out in the concentration ranges of 0.01322 to 0.01925 mg mL^{-1}, 0.003848 to 0.011544 mg mL^{-1}, 0.003926 to 0.011778 mg mL^{-1}, 0.007954 to 0.023862 mg mL^{-1} and 0.004022 to 0.012066 mg mL^{-1} respectively. The plots were linear over these concentration ranges (n = 7) for the reported drugs. The peak areas of Phenylephrine HCl, Cetirizine HCl, Loratadine HCl, Montelukast sodium and Ebastine were graphed against their respective concentrations. The linear regression analysis was carried out using the developed curves. The correlation coefficient (r) (n = 7) for Phenylephrine HCl, Cetirizine HCl, Loratadine HCl, Montelukast sodium and Ebastine were 0.9990, 0.9995, 0.9999, 0.9999 and 0.9990 respectively. The values of RSD and confidence levels (95%

confidence interval) were ranged from 0.481 to 1.433% and 100.104 to 101.732% in the concentration ranges considered.

5.3.2.2.2 Limits of Detection and Quantitation

The LOD and LOQ of Phenylephrine HCl, Cetirizine HCl, Loratadine HCl, Montelukast sodium and Ebastine were 0.8292 and 2.5126, 0.3750 and 1.1365, 0.1553 and 0.4705, 0.3403 and 1.0312 and 0.6542 and 1.9823µg mL^{-1}, correspondingly.

5.3.2.2.3 Specificity

The reported method was pretty specific. The retention times of all the molecules in the standard samples were comparable with those of the plasma samples. There was no affect of the supplemented impurities (in the standards) on the retention times and the peak shapes of these drugs. These results showed good specificity of the reported UFLC method.

5.3.2.2.4 Precision

The precision data was determined at three different concentrations i.e. 0.001, 0.05 and 0.10 mg mL^{-1} of all the reported molecules. Five sets of UFLC runs were carried out for all three concentrations. The values of RSD and confidence levels were ranged from 0.16 to 1.06% and 99.0 to 99.0%.

5.3.2.2.5 Accuracy

The accuracy of the chromatographic method was determined by the different concentrations of the reported molecules. Three concentrations used were 0.001, 0.05 and 0.10 mg mL^{-1}. UFLC experiments were done five times (n = 5). The

accuracies were ascertained by interpolation of five replicates peak areas of these drugs. The values of the absolute errors were ranged from 1.60 to 1.88%.

5.3.2.2.6 Robustness

The robustness of the chromatographic method was ascertained by altering slight variations in the UFLC experiments. The diverse experimental settings were solvent system composition, flow rate, temperature and wavelength. The retention time, peak area and shape were analyzed in the developed and slightly varied experimental conditions.

5.3.2.2.7 Ruggedness

The ruggedness of UFLC method was ascertained by altering the experimental conditions such as different operators and several days.

5.4. APPLICATION OF THE DEVELOPED SPE AND UFLC METHODS TO THE REAL WORLD SAMPLES:

The developed and validated SPE and UFLC methods were adopted for the determination of the reported drugs in the human plasma samples. The values for the retention, separation and resolution factors were ranged from 2.05 to 11.03, 1.16 to 2.33 and 1.03 to 6.10, correspondingly. These values were in good agreement with those obtained with the standard drugs. The peak heights were low in comparison to the standard solutions. It was due to the fact that some concentrations of the drugs interacted with plasma proteins. The validation data was close to those obtained with the standard drugs. It was seen that there was no additional peak in the chromatograms, showing fine selectivity of SPE method. Besides, the lack of any extra peak confirmed no new molecule and metabolic

product during stay of these drugs in plasma sample. The chromatographic results in terms of retention, separation, resolution factors and symmetry of the eluted peaks were similar to those of the standard samples. These results indicated that the reported SPE and UFLC methods were selective, efficient, rugged and reproducible.

5.5. CONCLUSION:

From the results discussed herein, it can be concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine in human plasma. There was no extra peak in the plasma samples, which established the selectivity of SPE method. Also there was no degradation of these drugs in plasma sample. The synchronized separation and identification of these five drugs are reported first time. The developed SPE and UFLC methods were applied successfully for the monitoring of these drugs in human plasma. Therefore, SPE and UFLC methods may be used for the determination of these drugs in any plasma sample.

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6.1. SUMMARY:

6.1.1 Diagnostic Relevance of the Research Work

Nowadays, most of the people are suffering from various types of diseases and problems, globally. Among them cardiovascular (heart related diseases) is a great concern to the society. The continuous increasing rate of antidiabetic and antihistamine pateints is also a challenging for all of us. Therefore, it is very essential to design and develop fast, inexpensive, selective, efficient and effective analytical methods for the analyses of these drugs using core shell porous columns and in different types of matrices. Among various analytical techniques chromatography is the best one due to its fast, effective and reproducible results. From literature survey it was observed that there are some HPLC methods available for the analyses of cardiovascular, antidiabetic and antihistamine drugs. It was observed that these methods suffer from certain drawbacks. The major drawback includes costly experimentations, costly consumption of chemicals, time consuming, non-ecofriendly and high limits of detection and quantification. In addition to this, sample preparation is an integral part of drugs before HPLC analyses. Literature survey indicates sample preparation using liquid-liquid extraction. This method has some serious limitations such as use of large amounts of costly chemicals, time consumption, non-ecofriendly and poor extraction efficiencies. In considering above fact, it was considered worldwide to develop most effective and ecofriendly SPE, HPLC and UFLC methods for analyses of cardiovascular, antidiabetic and antihistamine drugs for the good health of human beings.

The current research work includes separation and identification of some classes of drugs such as cardiovascular, antidiabetic and antihitamine. For this SPE, HPLC and UFLC methods were used and the results found to be fast, selective,

economic and reproducible. The details of the conclusion with chapter wise is given herein.

In *Chapter* 3, analyses of a series of cardiovascular drugs were carried out. The developed and validated SPE and HPLC methods for cardiovascular drugs separation in human plasma were compared with those reported in the literature. The comparison was carried out in terms of number of cardiovascular drugs separated, detection limit, flow rate and mobile phase. The values of retention, separation and resolution factors were ranged from 0.19-3.40, 1.20-3.60 and 2.43-12.37, respectively. The developed and validated SPE and HPLC methods were applied to the real world samples. The plasma sample residues were diluted wherever required. The reported SPE and HPLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of amiloride, metoprolol, hydrochlorothiazide, carvidilol, amlodipine, frusemide, telmisartan, losartan and olmesartan in human plasma. There was no extra peak in plasma samples, which confirmed no drug-drug interaction for the reported cardiovascular drugs. Besides, the absence of any new peak established no metabolic product. The separation and identification of these nine cardiovascular drugs is reported first time so far. Therefore, SPE and HPLC methods can be applied for analyses of these nine cardiovascular drugs. The developed SPE and HPLC methods were applied successfully for monitoring these drugs into human plasma.

In *Chapter* 4 analyses of anti-diabetic drugs were carried out. The results of this study were compared with those available in the literature. By comparing the results of this study with literature, it was concluded that the present work was the best with complete base line separation and lower limit of detection. The values of the retention, separation and resolution factors were ranged from 0.07 to 9.14, 1.44 to 4.21

and 2.15 to 18.66, correspondingly. The developed and validated SPE and UFLC methods were applied for monitoring anti-diabetic drugs into human plasma samples. The retention, separation, resolution factors and symmetry of the peaks were similar to those of the standard samples. In view of above facts, it can be concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of metformin HCl, vildagliptin, gliclazide, linagliptin, sitagliptin, pioglitazone, glimepiride and repaglinide in human plasma. There was no extra peak in the plasma samples, which established the selectivity of SPE method. Also there was no degradation of these drugs in plasma sample. The synchronized separation and identification of these eight drugs are reported first time. The developed SPE and UFLC methods were applied successfully for the monitoring of these drugs in human plasma. Therefore, SPE and UFLC methods may be used for the determination of these drugs in any plasma sample.

Similarly, In *chapter* 5, analyses of antihistamine drugs were carried out. The results of this study were compared with those available in the literature. The developed and validated SPE and UFLC methods were adopted for the determination of the antihistamine drugs in the human plasma samples. The values for the retention, separation and resolution factors were ranged from 2.05 to 11.03, 1.16 to 2.33 and 1.03 to 6.10, correspondingly. In view of these facts, it can be concluded that the reported SPE and UFLC methods were selective, efficient, rugged, economic, eco-friendly and reproducible for the separation and identification of phenylephrine HCl, cetirizine HCL, loratidine HCl, montelukast sodium and ebastine in human plasma. There was no extra peak in the plasma samples, which established the selectivity of SPE method. Also there was no degradation of these drugs in plasma sample. The synchronized separation and identification of these five drugs are reported first time. The developed

SPE and UFLC methods were applied successfully for the monitoring of these drugs in human plasma. Therefore, SPE and UFLC methods may be used for the determination of these drugs in any plasma sample.