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Simultaneous determination and validation of Chlorpheniramine Maleate, paracetamol, Phenylpropanolamine Hydrochloride and Caffeine in Solid Pharmaceutics by using reveres phase high performance liquid Chromatography (RP-HPLC).

A Thesis

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By

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Abstract

The simultaneous determination of Chlorpheniramine maleate (CPM), Paracetamol (PAR), Phenylpropanolamine hydrochloride (PPA) and Caffeine (CAF) was determined by Reversed phase High-Performance Liquid-Chromatography (RP-HPLC).The main advantages of this method are: simply, sensitive, rapid and accurate . The separation was achieved by using ODS2-C_{18} (250 mm x 4.6mm, 5µm par analytical column at ^{o}C; the mobile phase was acetonitrile- water - methanol- orthophosphoric acid (1M)- triethylamine and hexanesulfonic acid (14mmole L^{-1}) as ion pair with the volume ratio (v/v) of (15:75:10:0.5:0.3:4) ;respectively ,adjusting pH to 2.8 orthophosphoric acid ; flow programming was applied from 1mL/min (in 0-5 min) to 1.7 mL/min (after 5 min) till 8 min using UV detection a nm. The retention time of CPM, PAR, PPA and CAF were found to be (3.423, 5.627, 6.052 and 7.690) min; respectively. The validity of the proposed method was evaluated by determining the value of linearity, accuracy, recovery, precision, limit of detection, limit of quantitation and robustness. It was found that the values of linearity and correlation coefficient of developed method were (1-50) µg/mL (R^2 = 0.9999), (1-500) µg/mL (R^2 = 0.9999), (1-200) µg/mL (R^2 = 0.9998) and (1-150) µg/mL (R^2= 0.9998) for CPM, PAR, PPA and CAF; receptively. The validation methods provided excellent levels of precision and accuracy with lower range of R.S.D.% (< 1.5%) and perfect range of percentage recoveries (101.650-101.640)%, (100.305-99.484)%, (100.840-100.751)% and (98.075-100.461)%, for CPM, PAR, PPA and CAF, receptively. limit of detection and limit of quantitation were found to be (0.516, 0.425, 0.466 and 0.683) µg/mL and (1.720, 1.416, 1.552 and 2.277) µg/mL for CPM, PAR, PPA, and CAF; respectively. The effect of pH, temperature, volume injected and flow rate was also determined. pH (2.8) , Temperature (25^{o}C), volume injected (20 µl) were selected for the present work. The results in this study show that the proposed method was successfully applied to estimate the CPM, PAR, PPA and CAF in pharmaceutical preparation.

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List of symbols and abbreviation

symbol	***Title***
HPLC	High performance liquid chromatography
NP-HPLC	Normal phase – High performance liquid chromatography
RP-HPLC	Reversed phase – High performance liquid chromatography
IEC	Ion exchange chromatography
SEC	Size exclusion chromatography
K'	Capacity factor
α	Selectivity
p	Polarity
Ø	Volume fraction
t_R	Retention time
V_R	Retention volume
t_o	Dead time
V_o	Dead volume
F	Flow rate
t'_R	Reduced retention time
V'_R	Reduced retention volume
N	Number of theoretical plate
W	Width of peak
L	Length of column
H	Height of column
R_s	Resolution of separation
A_s	Asymmetric factor
TF	Tailing factor
B%	Organic solvent percent
i.d	Internal diameter

RI	Refractive index
PAR	Paracetamol
AMP	Acetaminophen
CAF	Caffeine
PPA	Phenylpropanolamine hydrochloride
CPM	Chlorpheniramine maleate
OTC	Over the counter
FAD	Food and Drug Administration
LOD	Limit of detection
LOQ	Limit of quantitation
R.S.D%	Relative standard deviation percent
S.D	Standard deviation
S	Slope of calibration
HSA	Hexanesulfonic acid
TEA	Triethylamine
P	Pseudoephedrine hydrochloride
FT-IR	Fourier transform infrared spectrophotometer
S.D.I	The State Company for Drugs Industry and Medical Appliance / Iraq
E%	Error percent
T_k	Temperature in Kelvin
DEA	Drug Enforcement Administration
USP	United State Pharmacopeia

Chapter one

General Introduction

(1-1) High performance liquid chromatography (HPLC)

Chromatography is a general term applied to a wide variety of separation techniques based on the sample partitioning between a moving phase, which can be a gas, liquid, or supercritical fluid, and a stationary phase, which may be either a liquid or a solid. The discovery of chromatography is generally credited to Tswett, who in 1906 described his work on using a chalk column to separate pigments in green leaves. The term ‘‘chromatography’’ was coined by Tswett to describe the colored zones that moved down the column. The technique languished for years, with only periodic spurts of development following innovations such as partition and paper chromatography in the 1940s, gas and thin-layer Chromatography in the 1950s, and various gel or size-exclusion methods in the early 1960s. Through the decade of the 1970s the technique grew with astounding speed, and for nine consecutive years from the middle 1970s to the early 1980s the technique led all other analytical instruments in growth rate in an annual survey conducted by Industrial Research and Development magazine [1].

(1-2) Modes of separation in HPLC

(1-2-1)Normal phase chromatography (NP-HPLC)

Normal-phase HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. In a mixture of components to be separated those analytes which are relatively more polar will be retained by the polar stationary phase longer than those analytes which are relatively less polar. Therefore the least

polar component will elute first. The attractive forces which exist are mostly dipole-dipole and hydrogen bonding (polar) interactions [2].

(*1-2-2) Reversed phase HPLC (RP -HPLC)*

RP-HPLC is the dominant HPLC mode, especially for the separation of mixtures of organic compounds [1]. In RP-HPLC, the column, packed with derivative silica particles, acts as a non-polar retaining stationary phase. Support-based stationary phases such as octadecyl (C_{18}), octyl (C_8), phenyl-hexyl (Ph- C_6) and cyano (CN) phases are commonly employed. The binary mobile phase is of mid to high polarity and consists of an aqueous phase, which may be slightly acidified, and an organic component. Acetonitrile and methanol are the preferred organic solvents, whilst isopropanol and tetrahydrofuran may also be used [3]. RP-HPLC provides separation according to hydrophobicity–analyte molecules partition between the non-polar stationary phase and the polar mobile phase, leading to increased retention of the less polar analytes [4].

(1-2-3) Ion exchange chromatography (IEC)

Ion-exchange chromatography, as indicated by its name, is based on the different affinities of the analyte ions for the oppositely charged ionic centers in the resin or adsorbed counter ions in the hydrophobic stationary phase [5].

The nature of functional groups providing the site for exchange, quaternary ammonium ($-N^+R_3$) for anion and sulphonic acid ($-SO_3H$) for cations is the same in HPLC as in classical ion exchange chromatography [6].

(1-2-4) Size exclusion chromatography (SEC)

SEC is the method for dynamic separation of molecules according to their size; as indicated by its name, the separation is based on the exclusion of the molecules from the porous space of packing material due to their steric hindrance. Hydrodynamic radius of the analyte molecule is the main factor determining its retention. In general, the higher the hydrodynamic radius, the shorter the retention [7].

Size-exclusion chromatography provides a rapid means for separating larger molecules, including polymers and biomolecules. Size-exclusion chromatography can be carried out using conventional HPLC instrumentation, replacing the HPLC column with an appropriate size-exclusion column. A UV/Visible detector is the most common means for obtaining the chromatogram [8].

(1-2-5) Affinity chromatography

Affinity chromatography is a modified gel technique which exploits the specificity of a donar-ligand interaction, many application involve bio specific binding between an immobilized biochemical and protein. The technique uses packing typically based on soft gel such as agarose the ligand being immobilized by covalent bonding to the matrix. Often short–chain alkyl group or spacer is inserted between the ligand and the matrix to eliminate steric hindrance of the binding interaction. Some sample is applied to the column in a suitable buffered eluant such that the substrate becomes specifically, though not irreversible bound to the ligand.

The composition of pH of the eluant is then altered in such manner to weaken the ligand-substrate bonding, thus promoting dissociation and facilitating elution of the retained compound. Due to the nature of the

technique packing are synthesized for particular application. Thus affinity chromatography, while outside the mainstream of HPLC, has considerable potential and utility in biochemical and clinical application, such as the purification of antigens, enzymes, proteins, viruses and hormones [6].

(1-3) Mobile phase

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation the least polar solute spends proportionally less time in the polar stationary phase and is the first solute to elute from the column. Retention times are controlled by selecting the mobile phase, with a less polar mobile phase leading to longer retention times. If, for example, a separation is poor because the solutes are eluting too quickly, switching to a less polar mobile phase leads to longer retention times and more opportunity for an acceptable separation. When two solutes are adequately resolved, switching to a more polar mobile phase may provide an acceptable separation with a shorter analysis time. In a reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. Increasing the polarity of the mobile phase leads to shorter retention times, whereas longer retention times require a mobile phase of lower polarity [9].

(1-4) Mobile phase optimization

Control of sample retention in adsorption chromatography is achieved almost exclusively by modifying the composition of the mobile phase. Minor changes in the eluant strength have dramatic effect on K' and α

value. However, often a single solvent is unable to effect resolution of the sample components the binary and ternary solvent most be employed. A measure of solvent strength of these mixtures may be determined from the solvent polarity indices.

The polarity index, *P*, is a numerical measure of the relative polarity of various solvents as determined from their solubility in some specific solvents. The polarity index P_{AB} for a mixture can then be readily determined from the polarity indices of the pure components and their respective volume fraction ($Ø_A$, $Ø_B$), thus

$$P_{AB} = Ø_A P_A + Ø_B P_B \qquad (1\text{-}1)$$

Any desired polarity index can be obtained by mixing the appropriate amount of solvents. An increase in polarity of the solvent mixture means a stronger eluant and hence smaller *K'* values. Often satisfactory resolution can be achieved by adjusting the capacity factor by modifying the solvent strength. If, however, separation is still incomplete due to $\alpha \Rightarrow 1$, then α can be increased by changing the chemical nature of the of the solvent while maintaining its polarity[6].

(1-5) Stationary phases

The column is the only device in the high-performance liquid chromatography (HPLC) system which actually separates an injected mixture. Column packing materials are the media producing the separation, and properties of this media are of primary importance for successful separations. Several thousands of different columns are commercially available, and when selecting a column for a particular separation the chromatographer should be able to decide whether a

packed, capillary, or monolithic column is needed and what the desired characteristics of the base material, bonded phase, and bonding density of selected column is needed. Four distinct characteristics could be used for column classification :

1. Type (monolithic; porous; nonporous)
2. Geometry (surface area; pore volume; pore diameter; particle size and shape; etc.)
3. Surface chemistry (type of bonded ligands; bonding density; etc.)
4. Type of base material (silica; polymeric; zirconia; etc)

Most geometry-related properties of packing materials are related to the column efficiency and flow resistance: particle size, particle shape, particle size distribution, packing density, and packing uniformity. Surface-chemistry-related properties are mainly responsible for the analyte retention and separation selectivity.

Adsorbent surface area, pore volume, and pore diameter are the properties of significant importance. HPLC retention is generally proportional to the surface area accessible for a given analyte surface area. Accessibility is dependent on the analyte molecular size, adsorbent pore diameter, and pore size distribution.

The chemical nature of the ligands bonded on the surface of support material defines the main type of chemical interactions of the surface with eluent and analyte molecules. In essence, all C_{18}-type columns should be similar with regard to their main interaction type, namely, hydrophobic interactions: methylene selectivity of all C_{18}-type columns is virtually identical. Bonded phases of the same type differ in their ability to suppress (or shield) other types of interactions (ionic; dipole) exerted by the base material (e.g., silica). Energy of these unwanted interactions is about 10 times greater than the energy of dispersive interactions. Due to

the exponential nature of the relationship between retention and interaction energy even the presence of 1% or less of these active centers in the packing material surface can significantly affect the analyte retention.

Bonding density is the primary parameter in evaluation of the quality of the bonded material. Usually the higher the bonding density, the better the shielding effect, although care should be taken in cross-evaluation of similar columns on the basis of their bonding density. Surface geometry can also significantly affect bonding density. Base material with smaller pores has higher surface area; however, bonding density is usually lower due to the smaller pores.

All parameters of the packing material are interrelated in their influence on the chromatographic performance of the column .The quality of an HPLC column is a subjective factor, which is dependent on the types of analytes and even on the chromatographic conditions used for the evaluation of the overall quality [10].

(1-6) HPLC descriptors

(1-6-1) Retention time (t_R)

The distance of the peak maxima from the injection point expressed in time units is called retention time (t_R), and it serves as an identifier for the given analyte on that particular system. Retention time is probably the most widely used descriptor of the analyte behavior, and it is the most easily measurable parameter. However, even though it is easily measurable, it is the least universal parameter. Analyte retention time is dependent on the mobile phase flow rate; the faster the flow rate, the smaller the analyte retention time. It is also dependent on the flow rate

stability. The product of the analyte retention time and the mobile-phase flow rate is the retention volume (V_R). Analyte retention volume is more universal descriptor of the analyte behavior in the chromatographic system, since it is less dependent upon the instrumental parameters [10].

(1-6-2)Dead volume (t_o)

Even if the analyte does not interact with the stationary phase, it will not appear in the detector immediately after injection. An HPLC column is filled with small particles of porous material which have a significant volume of the liquid phase between the particles and inside their porous space, so the non interacting analyte still has to travel through this volume before it enters the detector. The volume of the liquid phase in the column is called void volume (V_o). Several other names are also used in the chromatographic literature: dead volume, hold-up volume, and sometimes retention volume of non retained component. If a particular HPLC system provides constant and stable mobile-phase flow (F), one can convert retention volume (V_R) and void volume (V_o) in to the retention time (t_R) and a void time (t_o) [11].

$$V_R = F.\ t_R \qquad (1\text{-}2) \qquad , \qquad V_o = F.\ t_o \qquad (1\text{-}3)$$

(1-6-3) Capacity factor (K')

The analyte retention consists of two parts: (1) the time of the component resides in the mobile phase actually moving through the column and (2) the time of the analyte is retained on the stationary phase. The difference between the total retention time (t_R) and the hold-up time

is called the reduced retention time (t'_R), and corresponding difference between the analyte retention volume and the void volume is called the reduced retention volume, (V'_R).The ratio of the reduced retention volume to the void volume is a widely used dimensionless parameter called retention factor, k'.

$$K'_1 = \frac{v_R - v_o}{v_0} = \frac{v'_R}{v_0} = \frac{t_R - t_0}{t_0} \qquad (1\text{-}4)$$

$$K'_2 = \frac{v_R - v_o}{v_0} = \frac{v'_R}{v_0} = \frac{t_R - t_0}{t_0} \qquad (1\text{-}5)$$

Retention factor (sometimes called capacity factor) is a very convenient chromatographic descriptor since it is dimensionless and independent on the mobile phase flow rate and column dimensions [12].

(1-6-4) Selectivity

The ability of the chromatographic system to discriminate different analytes is called selectivity (α).Selectivity is determined as the ratio of the retention factors of two analytes, or the ratio of the reduced retention times

$$\alpha = \frac{k\prime_2}{k\prime_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \qquad (1\text{-}6)$$

The increase of the selectivity in the development of the separation of a complex mixture is the primary goal of any chromatographer, because if the selectivity for the pair of analytes is equal to 1, then it does not matter how narrow your peaks or how fast your separation you will not be able to separate these components until you increase the selectivity [13].

(1-6-5) Efficiency

Early papers on chromatographic theory described the technique in terms similar to distillation or extraction. The Nobel Prize-winning work by Martin and Synge in 1941 introduced liquid–liquid (or partition) chromatography and the accompanying theory that became known as the plate theory. Although the plate theory was useful in the development of chromatography, it contained several poor assumptions. The theory assumed that equilibration between phases was instantaneous, and that longitudinal diffusion did not occur. Furthermore, it did not consider dimensions of phases or flow rates. An alternative to the plate theory which came into prominence in the 1950s was the so-called rate theory.
The paper which has had the greatest impact was published by the Dutch workers van Deemter, Zuiderweg, and Klinkenberg . They described the chromatographic process in terms of kinetics and examined diffusion and mass transfer. The popular van Deemter plot resulted, and the rate theory has become the backbone of chromatographic theory.

The width of a band or peak is a measure of the efficiency of the process whilst resolution is assessed by the ability to resolve the peaks of components with similar (t_R) or (R_f) values.

Efficiency, N (number of plate), for column separations is related to retention time and peak width measured in terms of the standard deviation, assuming an ideally Gaussian-shaped peak

$$N = \left(\frac{t_R}{w}\right)^2 \qquad (1\text{-}7)$$

In practice it is easier to measure baseline width or the width at one half of the peak height, so N is generally calculated using one of the alternative formulae:

$$N = 16\left(\frac{t_R}{w}\right)^2 \qquad (1\text{-}8)$$

Or $$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \qquad (1\text{-}9)$$

Where W is the baseline peak width and $W_{1/2}$ is the peak width measured at half of the peak height. Valid comparisons of efficiencies can be made only if the same formula is used throughout, as the computed values of N using each of the above formulae may differ considerably.

The parameter N is universally referred to as the plate number, but an alternative means of quoting efficiency is in terms of a plate height, H.

$$\mathrm{N} = \frac{L}{H} \qquad (1\text{-}10)$$

Where (L) is the length of the column. Values of N may be many thousands for columns having high efficiencies, the corresponding values of H being less than 1 mm [6].

(1-6-6) Resolution of separation(R_s)

The distance between the peak maxima reflects the selectivity of the system. The greater the distance, the higher the selectivity. The width of the chromatographic peak reflects the system band broadening and thus efficiency. Resolution, R_s, is defined [equation (1-10)] as the ratio of the distance between two peaks to the average width of these peaks (at

baseline), and this descriptor encompasses both the efficiency and selectivity.

$$R_s = 2\frac{t_{R,2} - t_{R,1}}{w_{2+}\ w_1} \qquad (1\text{-}11)$$

For the resolution of a so-called “critical pair "of analytes (two analytes in the mixture that have minimal distance between them compared to all other analytes in the mixture), if they have relatively high retention factors (k′ >5) that their peak widths can be assumed as equal, then equation (1-9) reduces to

$$R_s = \frac{t_{R,2} - t_{R,1}}{w} \qquad (1\text{-}12)$$

Peak width could be expressed as:

$$w = \frac{4t_R}{\sqrt{N}} \qquad (1\text{-}13)$$

If we select the retention of the second analyte for the calculation of the peak width, then applying equation (1-12) into expression (1-11) we get:

$$R_s = \frac{t_{R,2} - t_{R,1}}{t_{R,2}} \cdot \frac{\sqrt{N}}{4} \qquad (1\text{-}14)$$

Relatively simple algebraic conversion will bring us to so-called Master Resolution [Equation (1-15)] [13]:

$$R_s = \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1+ k_1} \cdot \frac{\sqrt{N}}{4} \qquad (1\text{-}15)$$

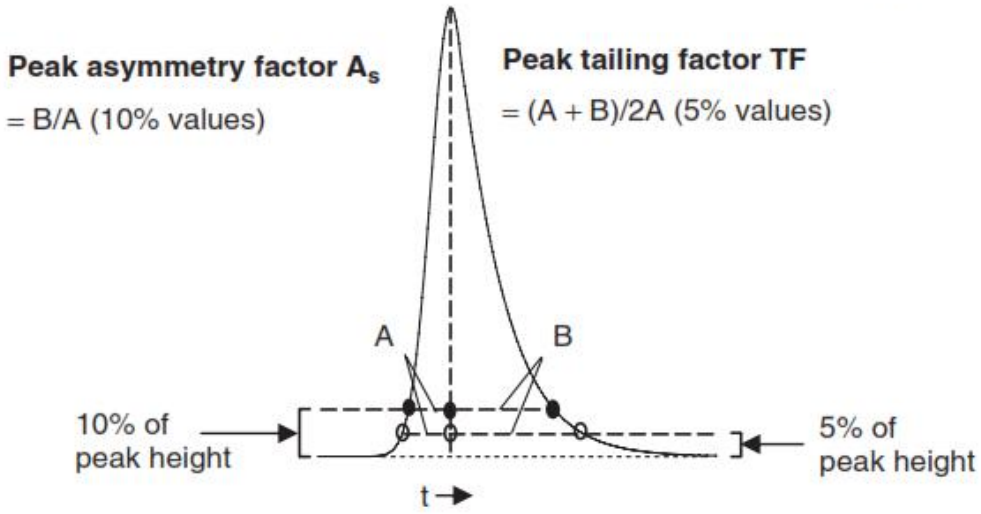
(1-6-7) Peak shape

Actual peaks in a chromatogram will usually depart slightly from a symmetrical, Gaussian shape, typically showing more or less tailing. Peak

tailing can be characterized in either of two ways (Figure 1-1): by the asymmetry factor A_s (left-hand side of figure) or by the tailing factor TF (right-hand side). Values of A_s and TF are related approximately as

$$A_s \approx 1 + 1.5(TF-1) \qquad (1\text{-}16)$$

So that values of A_s are typically somewhat larger than values of TF [3].



(Fig.1-1): measurement of peak asymmetry A_s and peak tailing TF

(1-7) Method development[3]

The steps involved in method development:

(1-7-1) Assessment of sample composition and separation goals.

At the start of method development, available information about the chemical composition of the sample should be reviewed. If acidic or basic compounds are present, it will be necessary to add a buffer to the mobile

phase so as to control its pH; pKa values for sample compounds, if available, can be useful during method development. The molecular weight of the sample may also affect the choice of separation conditions for initial experiments. Samples that contain enantiomers will require the development of a special method for their separation.

The separation goals for a sample may not be limited to adequate resolution and minimum run time. Depending on the equipment that is available for the routine application of the final method, gradient elution may not be possible (an isocratic method will therefore be required). Trace analysis, as in the determination of compound impurities, may impose additional requirements on both sample preparation and the detector. Quantitative analysis calls for some minimum precision [e.g.,±(1–2)% for major components and ±(10–20)% for trace constituents]. However, often only some of the sample components will require separation; for example, drugs present in blood or urine, or pesticides in water or soil samples.

(1-7-2) Sample pretreatment

Prior to injection the sample may require some processing in order to remove components that can damage the column or interfere with the separation of compounds of interest. Sample pretreatment procedures involve multiple steps and use of a wide range of separation media. For this reason these procedures can be more difficult to develop than the subsequent HPLC separation. When a pretreatment procedure for a similar combination of analyte and sample matrix is available (either as developed in the same laboratory or as reported in the literature), its use or adaptation for the sample is often preferred.

(*1-7-3) Selection of chromatographic mode*

Reversed-phase chromatography is the default choice for HPLC method development. However, depending on the sample, other chromatographic modes may be preferable. Often times this does not become apparent until initial experiments with RP-HPLC prove unsuccessful. Similarly, if isomeric compounds are present in the sample and prove difficult to separate by RP-HPLC, the use of NPC with unbonded silica as column packing will often prove more successful. NPC with unbonded silica is also preferred for preparative-scale separations .

(1-7-4) Detector selection

Choice of the proper detection scheme is dependent on the properties of the analyte. Different types of detectors are available (As will be explained later), However, the majority of reversed-phase and normal-phase HPLC method development in the pharmaceutical industry is carried out with UV detection. A wavelength for UV detection must be chosen so that an accurate mass balance may be determined. Therefore, if area% normalization is to be used, then all the impurities and the active pharmaceutical ingredient must have similar relative response factors (area response/weight).This is sometimes difficult because the impurities may have different electron-donating or electron-withdrawing functional groups, attached to the aromatic ring and/or the impurities may have more complex conjugated systems and the absorption spectra have been shifted to longer or shorter wavelengths compared to the parent compound. Therefore the UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to

different amounts present in the mixture. A wavelength must be chosen such that adequate response is obtained for the active and that at least a (0.05%v/v) solution of the active at target concentration could be quantified . The wavelength chosen should not be on a distinct slope of the spectrum, and the relative difference in the absorbance at a certain wavelength is not significantly different from the impurities/degradation products present [11].

(1-7-5) Choice of separation conditions

For most samples, a systematic, trial-and-error approach can be followed, based on three successive steps. First, mobile-phase strength (B%) (Organic solvent) is varied until the right retention range is achieved, for example, $1 \leq k' < 10$. Second, different separation conditions are explored for acceptable selectivity (values of α) and resolution. The first conditions that should be explored for improved selectivity are changes in B% (e.g., ±10%B) and temperature [e.g., (30–50) ˚C]. If some peaks are still overlapped and poorly separated, other conditions can be varied to improve selectivity. The third step is to vary column conditions: column length, particle size, and/or flow rate. A change in column conditions can provide a moderate increase in the plate number N and resolution, usually at the expense of a longer separation time (run time). When the sample resolution is better than necessary ($R_s >> 2$) after optimizing selectivity, a reduction in column length and/or an increase in flow rate can result in a much shorter run time. In many cases, adequate separation can be achieved within a day or two, based on a small number of experiments [3].

(1-8) Instrumentation

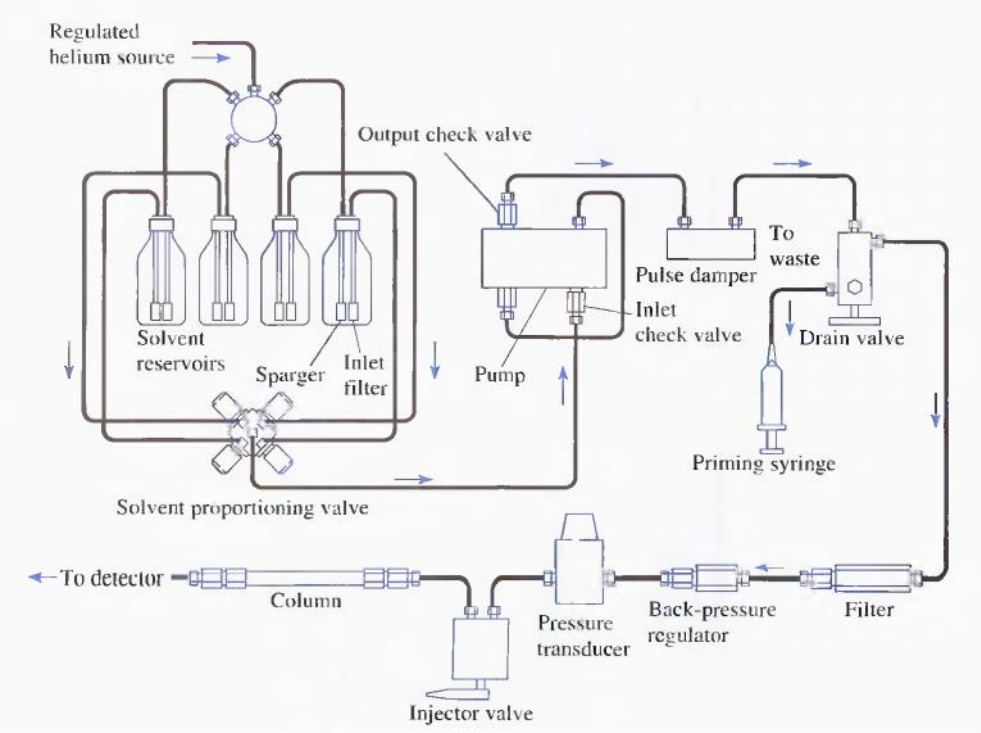


Fig. (1-2): Diagram showing components of a typical apparatus for HPLC system [14].

(1-8-1) Pumps

As a consequence of the large peak back pressures encountered, due to the small particle size of packing used in HPLC column , pumps must be employed to achieve acceptable eluant flow rates. The pumps may be classified as either those which provide constant inlet pressure or those which provide constant outlet flow and should be capable of delivering up to 7000psi (48.3 Mpa). They are constructed from materials which are resistance to the organic solvents and aqueous buffer solution commonly used as eluants. The system may also have provision for microprocessor control of the pumps to provide for complex gradient elution using high or low pressure mixing [15].

(1-8-2) The column

Columns are made from straight lengths of precision-bore stainless-steel tubing with a smooth internal finish. Typically they are (10–25) cm long and (4–5) mm i.d. Microbore columns, (20–50) cm long and with an i.d. of (1–2) mm, are sometimes used where sample size is limited and to minimize solvent consumption because the volumetric flow rate through them is less than a quarter of that through conventional columns. The stationary phase or packing is retained at each end by thin stainless-steel frits or mesh disks of 2 μm porosity or less. Columns are packed by a slurry method which involves suspending the particles of packing in a suitable solvent and 'slamming' it into the column rapidly and at pressures in excess of 3000 psi (200 bar).Column life is generally six months or more and can be prolonged by use of a guard column and a scavenger column. The former consists of a very short length of column placed between the injection port and the analytical column to trap strongly retained species or particulate matter originating in the mobile phase, the samples or from wearing of the injection valve. It is packed with relatively large particles (30 μm) of the same or a similar stationary phase to that used in the analytical column and requires periodic renewal. Scavenger columns are short lengths of tube packed with large particle silica and positioned between the pump and the injection valve with the principal object of saturating an aqueous mobile phase with silica to reduce attack on the packing in the analytical column, especially by high or low pH buffers [1].

The column consists of a cylindrical tube that is typically filled with small (usually 1.5- to 5μm diameter) spherical particles Figure (1-3) [3].

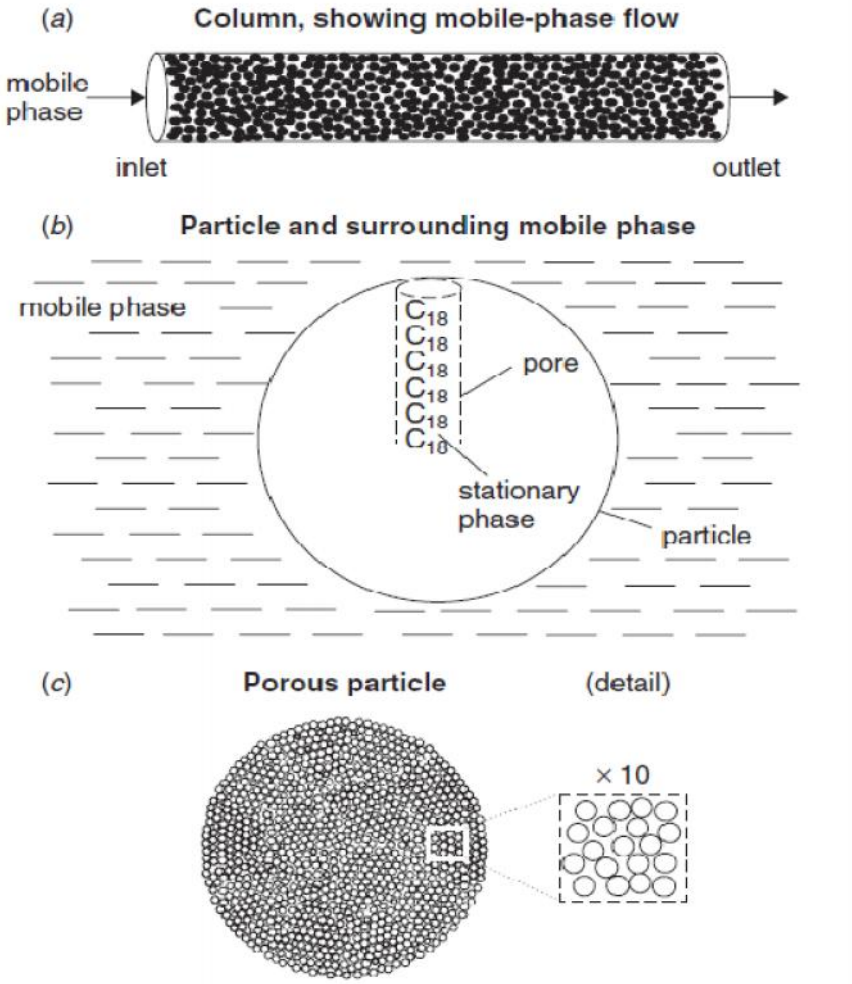


Fig.(1-3): The HPLC column. (a) Column packed with spherical particles ;(b) Schematic of an individual particle, showing an idealized with attached C_{18}group ;(c) More realistic picture of a spherical, porous particle, showing details (10 x expansion) [3].

(1-8-3) Detectors

The ideal HPLC detector should have rapid and reproducible response to solutes, a wide range of linear response, high sensitivity and stability of operation. No truly universal HPLC detector has yet been developed but the two most widely applicable types are those based on the absorption of UV or visible radiation by the solute species and those

which monitor refractive index differences between solutes dissolved in the mobile phase and the pure mobile phase. Other detectors which are more selective in their response rely on such solute properties as fluorescence, electrical conductivity, diffusion currents (amperometric) and radioactivity [16].

(1-8-3-1) UV/Visible photometers and dispersive spectrophotometers

These detectors respond to UV/visible absorbing species in the range (190–800) nm and their response is linear with concentration, obeying the Beer-Lambert law. They are not appreciably flow or temperature sensitive, have a wide linear range and good but variable sensitivity. Photometers are designed to operate at one or more fixed wavelengths only,(e.g. 220, 254, 436 and 546) nm, whereas spectrophotometers facilitate monitoring at any wavelength within the operating range of the instrument. Both types of detector employ low-volume (10 µl or less) flow-through cells fitted with quartz windows. Careful design of the cell, which should be of minimal volume to reduce band spreading, and maximal path length for high sensitivity, is necessary to reduce undesirable refraction effects at the cell walls as solutes pass through. Although many substances absorb appreciably at 254 nm or one of the other fixed wavelengths available with a photometer, a much more versatile detection system is based on a spectrophotometer fitted with a grating monochromater and continuum source, e.g. a deuterium lamp for the UV region and a tungsten-halogen lamp for the visible region (Figure 1-4). They have double beam optics, stable low-noise electronics and are often microprocessor controlled. Some can be programmed to select a sequence of optimum monitoring wavelengths during or between chromatographic runs, and the recording of a complete UV spectrum after

stopping the flow with a selected peak in the detector cell is a feature of other designs.

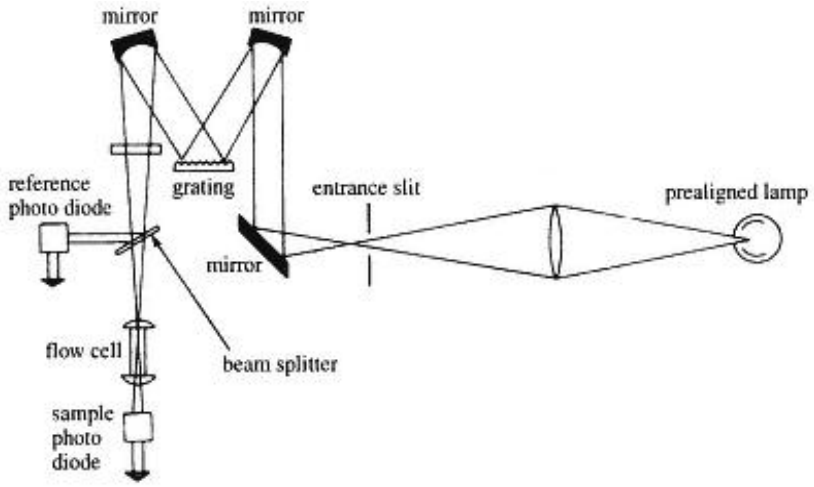


Fig.(1-4): Variable-wavelength detector, showing deuterium lamp, optical path, reference photodiode and monochromator.

Another development is a rapid scanning capability that allows a complete spectrum to be recorded in a fraction of a second without the need to stop the flow, therefore rivaling diode array detectors. Like the latter, full computer control and high resolution colour graphics enable chromatograms to be displayed in 3D and other formats and peak purity assessed. Sensitivity and resolution are better than some diode array detectors. Photometers are more sensitive than spectrophotometers, are cheaper and more robust and are well suited to routine work where monitoring at 254 nm or some other fixed wavelength is acceptable. Spectrophotometers, however, allow 'tuning' to the most favorable wavelength either to maximize sensitivity for a particular solute or to 'detune' the response to other solutes. By allowing monitoring down to 190 nm, weakly absorbing or saturated compounds can be detected [16].

(1-8-3-2) Diode array spectrophotometers

The diode array detector, although offering detection over a range of UV wavelength, functions in an entirely different way from that of the dispersive instrument. A diagram of a diode array detector is shown in figure (1-5).

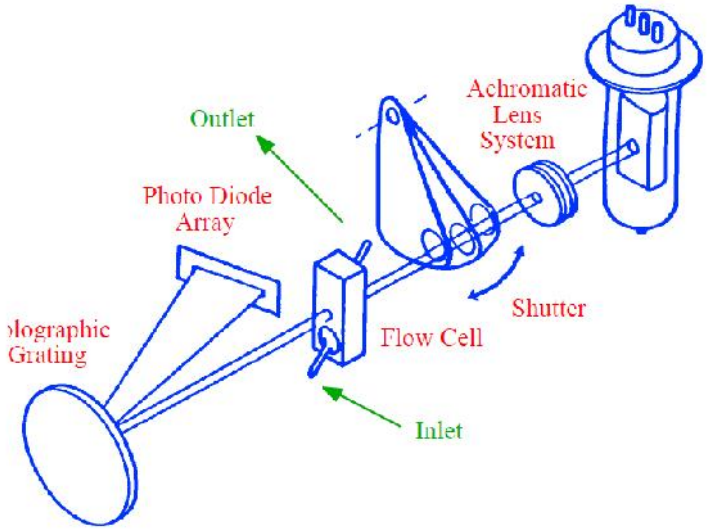


Fig. (1-5): Optical path in a UV photodiode array detector .

Light from a broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way the sample is subjected to light of all wavelengths generated by the lamp. The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc. At the end of the run, the output from any diode can be selected and a chromatogram produced employing the UV wavelength that was falling on that particular diode. Most instruments will permit the monitoring of a

least one diode in real time so that the chromatogram can be followed as the separation develops. This system is ideal in that by noting the time of a particular peak, a spectrum of the solute can be obtained by recalling from memory the output of all the diodes at that particular time. This gives directly the spectrum of the solute [17].

(1-8-3-3)Fluorescence detectors

Fluorescence is a luminescence phenomenon that occurs when a compound absorbs radiation, UV or visible, and then emits it at a longer wavelength. There are relatively few drugs that have such strong native fluorescence. For these compounds, fluorescence detection can usually achieve increased specificity and sensitivity over that obtained with UV detection [18].

(1-8-3-4)Refractive index (RI)

There are several types of RI detector, all of which monitor the difference between a reference stream of mobile phase and the column effluent. Any solute whose presence alters the refractive index of the pure solvent will be detected, but sensitivity is directly proportional to the difference between the refractive index of the solute and that of the solvent. At best they are two orders of magnitude less sensitive than UV/visible detectors. All RI detectors are highly temperature-sensitive, and some designs incorporate heat exchangers between column and detector to optimize performance. They cannot be used for gradient elution because of the difficulty in matching the refractive indices of reference and sample streams.

RI detectors are highly useful for preparative chromatography, where they can be operated at low sensitivity, and for polymer or macromolecular separations, where the change in refractive index from that of the mobile phase will be great, even for low concentrations of solute [19] The most common type of RI monitor is the deflection refractometer (Figure 1-6).

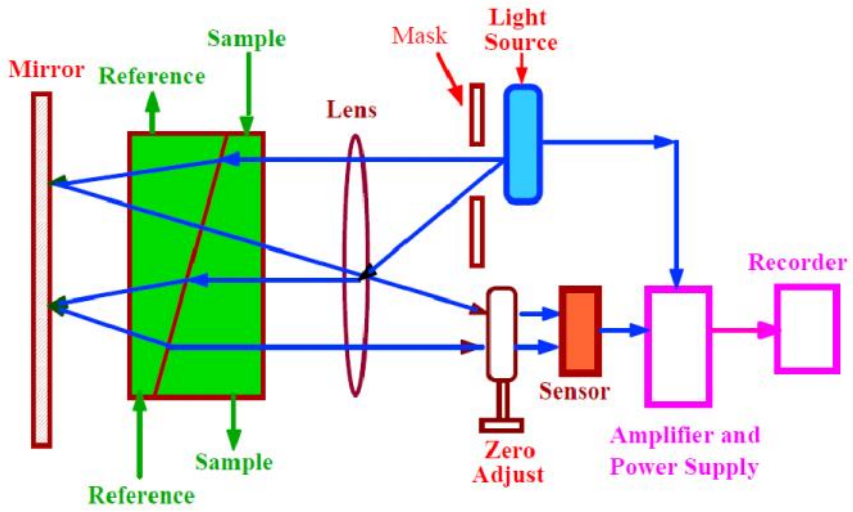


Fig. (1-6): Refractive index (RI) detector [17].

(1-8-3-5) Electrochemical detectors

Conductance monitors can be used where the sample components are ionic and providing that the conductivity of the mobile phase is very low. They are used extensively in ion chromatography for the detection of inorganic anions, some inorganic cations and ionized organic acids. Amperometric detectors, which are based on the principle of polarography, rely on measuring the current generated in an electrochemical cell at a fixed applied potential by the facile oxidation or

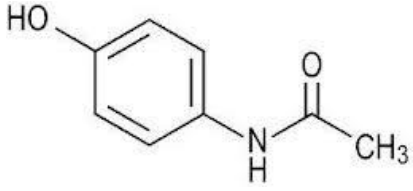
reduction of an eluted compound at the surface of a glassy carbon, gold or platinum micro-electrode. The cell is completed with a calomel reference electrode and an auxiliary electrode, the purpose of the latter being to enable the applied potential to be stabilized when the cell resistance alters by virtue of the currents generated. The mobile phase acts as a supporting electrolyte for the redox reactions and its composition is therefore restricted to predominantly aqueous solvent mixtures. Several designs have been produced, some with internal cell volumes as little as 1 µl. Amperometric detectors are amongst the most sensitive available but they are susceptible to noise, caused by any residual pulsations from the pump affecting the flow of mobile phase, and to surface contaminations of the micro-electrode due to the build-up of electrode reaction products which impairs reproducibility. However, their high sensitivity and selectivity (through variation of the applied potential) enhances their value for the trace analysis of certain types of compound [20].

(1-9)The drugs

(1-9-1)Paracetamol (PAR)[Acetaminophen (AMP)]

It is a widely used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer). It is commonly used for the relief of headaches, other minor aches and pains, and is a major ingredient in numerous cold and flu remedies [21,22].

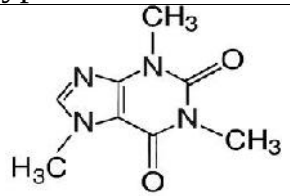
General property [23]:-

Chemical name	N-(4-hydroxyphenyl) acetamide
Molecular structure	$C_8H_9NO_2$
Molecular weight	151.17 g/mole
Solubility	sparingly soluble in water, freely soluble in alcohol, very slightly soluble in ether and in methylene chloride
Appearance	White, crystalline
PK_a	9.51
Melting point	(168-172) ^oC
Uses	Acetaminophen (also known as paracetamol, PAR), a well established analgesic/antipyretic drug, is frequently used by itself OTC (Panado, Tempra, Tylenol) or in combination with codeine (Tylenol 3), hydrocodone (Vicodin), or oxycodone (Percocet) for the treatment of mild to moderate pain and to reduce fever.
Structural form	

(1-9-2) Caffeine(CAF).

Caffeine has pharmaceutically important chemical properties. Caffeine is weak Brønsted-Lowry base. Caffeine cannot donate a proton from position7 and does not act as a Brønsted acid at pH values less than 14. Caffeine does have electrophilic sites at positions 1, 3, and 7. Caffeine in blood is not highly protein bound. Differences in the substituent at the 7-position may be involved. Additionally, caffeine is lipophilic and reputedly achieves higher brain concentrations. The half-life of caffeine is 5 to 8 hours. About 1% of Caffeine is excreted unchanged. The compound is metabolized in the liver. The major metabolite is 1-methyluric acid [22].

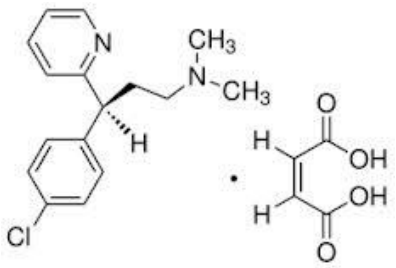
General property [24,25] :-

Chemical name	1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione
Molecular structure	$C_8H_{10}N_4O_2$
Molecular weight	194.1906 g/mole
Solubility	Sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol and ether. It dissolves in concentrated solution of alkali benzoate or salicylates
Appearance	crystalline powder or silky, white crystals, sublimes readily
pK_a	14 and 0.6
Melting point	(234-239) ^oC
Uses	A central nervous system stimulans, having the effect of temporarily warding off and restoring alertness, are effective for the acute treatment of tension type headache.
Structural form	

(1-9-3) Chlorpheniramine maleate(CPM).

Chlorpheniramine (Maleate) is the maleate salt of Chlorpheniramine. Chlorpheniramine is an antihistaminic agent derived from pheniramine. Chlorpheniramine is an anticholinergic antihistamine. [22].

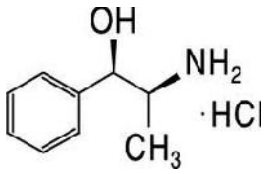
General property [26]:-

Chemical name	,(±)2-[p-chloro-[2-dimethylamino)ethyl] benzyl] pyridine bimaleate (Chlor-Trimeton)
Molecular structure	$C_{20}H_{23}ClN_2O_4$
Molecular weight	390.9 g/mole
Solubility	freely soluble in water , soluble in alcohol and slightly soluble in ether
Appearance	crystalline powder
PK_a	9.1
Melting point	(132-135) ^oC
Uses	It also is effective against nausea and motion sickness, with its primary mechanism of action being its ability to reduce acetylcholine levels in the brain. Acetylcholine is a neurotransmitter. In the brain, acetylcholine and dopamine have antagonistic effects on each other, so a proper balance is necessary for a healthy, functioning body and mind
Structural form	

(1-9-4) Phenylpropanolamine hydrochloride (Propadrine)(PPA).

Phenylpropanolamine hydrochloride was a common active component in OTC appetite suppressants and cough and cold medications until 2001 when the Food and Drug Administration (FDA) recommended its removal from such medications, because studies showed an increased risk of hemorrhagic stroke in young women who took the drug [27].

General property [18]:-

Chemical name	2-Amino-1-phenyl-1-propanol hydrochloride
Molecular structure	$C_9H_{14}ClNO$
Molecular weight	187.67 g/mole
Solubility	Freely soluble in water and in alcohol, practically insoluble in methylene chloride.
Appearance	White or almost white, crystalline powder.
PK_a	9.44
Melting point	(194-197) ^oC
Uses	Phenylpropanolamine hydrochloride is a psychoactive drug of the phenethylamine and amphetamine chemical classes which is used as a stimulant, decongestant, and anorectic agent. It is commonly used in prescription and over-the-counter cough and cold preparations. Phenylpropanolamine acts as a potent and selective releasing agent of norepinephrine and epinephrine, or as a norepinephrine releasing agent (NRA). It also acts as a dopamine releasing agent (DRA) to a lesser extent. It works by mimicking the effects of endogenous catecholamine such as epinephrine and norepinephrine and to a lesser degree dopamine.
Structural form	

(1-10) Aim of this study

1. Develop analytical method for the separation and determination of CPM, PAR, PPA and CAF.
2. Validate the developed method through the use of linearity, LOD, LOQ, R.S.D% and percentage recovery in order to establish quality control (precision and accuracy) values.
3. Investigate whether the developed method can be used as useful method in the determination of pharmaceutics.

Literature review for High-Performance Liquid Chromatographic (HPLC) Technique

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous estimation of Paracetamol and Naproxen in tablet formulation.	1 mL/min	3.005 for PAR.	99.72 for PAR.	0.7027 for PAR.	(5-80) µg/mL for PAR.	water(PH=2.5 adjusted with orthophosphoric acid : acetonitrile (87:13), C_{18} column (150mmx4.6mm i.d,5 µm),263nm.	[28]
Determination of Etoricoxib and Paraceta mol in pharmaceutical dosage form.	1 mL/min	8.34 for PAR.	101.8 - 100.4 for PAR at various added concentration	0.3 for PAR.	(48-146) µg/mL , LOD 0.973 µg/mL for PAR.	buffer(1 mL tifluro acetic acid in 2000 mL water: acetoni -trile(75:25),C_{18} column (25 cmx4.6, 5µm), 220 nm.	[29]
Determination of Phenylpropanolamine in pharmaceutical OTC preparations.	1 to 1.5 mL/min	4.7 for PPA	102.5 - 99.8 for PPA at various added concentration	2.2 to 1.2 for PPA at various added concentration	(2.5-1000) µg/mL for PPA	methanol: acetonitrile : acetic acid(0.1M) : triethylamine (20:20:60: 0.6 v/v/v/v) containing hexanesulfonic acid(0.5 mM) as ion pair, ODS column (250mm x 4.6mm ,5µm) ,254 nm.	[30]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Condition	Ref.
Determination of Paracetamol and Caffeine in tablet formulation.	1 mL/min	3.2 for PAR 5.26 for CAF.	100.22 - 101.97 for PAR at various added concentration 99.98 - 100.63 for CAF at various added concentration	0.59 for PAR 0.47 for CAF	(50-250) µg/mL , LOD 0.087 µg/mL for PAR. (5-25) µg/mL, LOD 0.654 µg/mL for CAF.	methanol: buffer (equal volume of 0.01 M orthophosphoric acid and 0.01 M monobasic sodium PH=4.2) (70:30) ,C_{18} column (150mm x 4.6 ,5µm),254nm.	[31]
Analysis of Paracetamol and Tramadol in a pharmaceutical dosage form.	1 mL/min	3.9 for PAR.	98.0 - 102 for PAR.	0.16 for PAR.	(20.8-39.0) µg/mL for PAR.	water with orthophosphoric acid : methanol (60:40 , v/v),C_{18} column (250mm x 4.6mm,5µm), 228nm.	[32]
Simultaneous quantitation of Paracetamol and Dexketoprofen Tromet-amol in bulk drug and formulation	1 mL/min	3.29 for PAR.	98.82 - 100.35 for PAR at various added concentration	1.528 - 1.321for PAR at various added concentration	(3-8) µg/mL ,LOD 0.1µg/mL for PAR.	methanol: ammonium acetate buffer (65:35), ODS– C_{18} column (250mm x 4.6mm ,5µm), 256nm.	[33]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Condition	Ref.
Determination of Chlorpheniramine Maleate and Phenylephrine in pharmaceutical dosage form	1 mL/min	3.31 for CPM.	101.09 - 98.99 for CP at various added concentration	0.50 for CP at various added concentration	(10-70) µg/mL , LOD 0.15µg/mL for CP.	acetonitrile: phosphate buffer (PH=5.6 adjusted by triethylamine) (55:45), C_{18} column (250mm x 4.6 mm, 5µm), 255 nm.	[34]
Determination of Aceclofenac, Paracetamol and Chlorzoxazone	1 mL/min	3.40 for PAR.	99.57 for PAR.	1.23 for PAR.	(25-75) µg/mL ,LOD 22ng/mL for PAR.	KH_2PO_4 : acetonitrile (60:40 v/v) pH adjusted to 5.55 with NH_4OH, C_{18} column (250mm x 4.6 mm ,5µm),205 nm.	[35]
Simultaneous quantitation and validation of Paracetamol, Phenylpropanolamine Hydrochloride and Cetirizine hydrochloride by in bulk drug and formulation	1 mL/min	3.10 for PAR 4.00 for PPA .	99.91 for PAR 100.01 for PPA at various added concentration	0.25 - 1.21 for PAR at various added concentration 1.62 - 1.49 for PPA at various added concentration	(0.4-1.4) µg/mL, LOD 0.2µg/mL for PAR . (7-12) µg/mL, LOD 0.4µg/mL for PPA.	methanol: disodium hydrogen phosphate dehydrate buffer (PH=7 adjusted with phosphoric acid) (60:40), C_{18} column (250mm x 4.6 mm, 5µm), 217 nm.	[36]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Analysis of Caffeine Paracetamol and its degradation Product p-aminophenol	1 mL/min	3.088 for PAR 7.607 for CAF.	100.48 for PAR 99.2 for CAF.	< 1.22	(0.08-0.8) mg/mL for PAR. (0.01-0.8) µg/mL for CAF.	methanol: phosphate buffer (PH=6) (20:80 v/v), C_{18} column (150mm x 4.6 mm ,5µm), 230 nm.	[37]
Simultaneous determination of Paracetamol, Phenylephrine HCl, and Chlorpheniramine Maleate in pharma-ceutical dosage Forms	1.5 mL/min	1.13 for PAR 3.44 for CPM.	99.99 for PAR 99.60 for CPM.	0.13 for PAR 1.36 for CPM.	(5-120) µg/mL for PAR. 0.2-3 µg/mL for CPM.	phosphate buffer (1.36 ml orthophosphoric acid in 1L water) (pH=6.22): acetonitrile (22:27) ,CN RP column (124A, 10 µm,3.9 x 150),165 nm .	[38]
Analysis of Paracetamol, Caffeine and Dipyrone	1 mL/min	4.88 for PAR 5.845 for CAF.	105.28 for PAR 49.43 for CAF.	2.02 for PAR 2.51 for CAF	(25-400) µg/mL, LOD 0.409µg/mL for PAR. (12.5-200) µg/ml,LOD 0.151µg/mL for CAF.	KH_2PO_4: methanol: acetonitrile: isopropyl alcohole (420:20:30:30), C_{18} column (250mm x 4.6 mm, 5µm), 215 nm.	[39]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous determination of Paracetamol,Phenyle-phrine Hydrochloride, Oxolamine Citrate and Chlorpheniramine-Maleate inpharma – ceutical dosage Forms	1.5 mL/min	1.18 for PAR 3.34 for CPM	99.99 fot PAR 99.60 for CPM.	0.12 for PAR 0.59 for CPM.	(20-120) µg/mL for PAR. (0.16-0.96) µg/mL for CPM.	(0.02 M)phosphate buffer(pH=4) : acetonitrile (85:15 v/v), SB CN (150mm x 4.6 mm ,5µm), 365 nm	[40]
Simultaneous determination of Paracetamol, Caffeine and Acetylsalicylic acid in Excedrin tablets	1 mL/min	5.40 for PAR 7.02 for CAF.	96.52 - 100.20 for PAR at various added concentration 96.50 - 100.40 for CAF at various added concentration	0.94 - 1.60 for PAR at various added concentration 1.40 - 1.59 for CAF at various added concentration	5-10x10^3 ng/ 10 µL ,LOD 4.88 g/10µL for PAR. 5-10^3 ng/ 10 µL, LOD 78.13 ng / 10 µL for CAF.	acetonitrile: NaH_2PO_4 (pH=3) (14:86), Caltrex (250x4.6 mm,5µm), 214 nm .	[41]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Quantification of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations: capsules and sachets.	1 mL/min	6.87 for CPM	100.00 for AMP 100.00 for CP.	0.56 for AMP 2.2 CP.	(0.154-0.461) mg/mL for AMP. (1.30-3.89) µg/mL for CPM.	methanol (with phosphate buffer, pH=6) : Acetonitrile (gradient elution). (250mm x4.6 mm, 5µm), 215 and 280 nm.	[42]
Determination of paracetamol and ascorbic acid in tablet dosage forms	1 mL/min	6.09 for PAR.	99.02 for PAR.	0.46 min for PAR.	(8.25-66.0) µg/mL ,LOD 0.5µg/mL for PAR.	0.4 ml formic acid: 25ml methanol: 75 mL water, C_{18} column (250 x 4.6 mm ,5µm),254 nm.	[43]
Simultaneous determination of Etodolac and Acetaminophen in tablet dosage form.	1 mL/min	4.24 for AMP	100.9 - 101.3 for AMP. at various added concentration	1.05 -0.25 for AMP at various added concentration	(120-280) µg/mL for AMP.	orthophosphoric acid (0.05%): acetonitrile (50:50) , C_{18}(100 mm x 4.6 mm , 5µm) , 254 nm .	[44]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous analysis of Codeine Phosphate, Ephedrine HCl and Chlorpheniramine maleate in cough/cold syrup formulation	1.5 mL/min	6.78 for CPM.	100.58 for CPM.	0.33 for CPM.	(50-120) µg/mL for CPM.	methanol/glacial acetic acid /triethylamine (980:15:6 v/v) and water/glacial acetic acid/triethylamine (980:15:6 v/v), C_8 column (150mm x 4.6 mm, 5µm), 254 nm .	[45]
Simultaneous determination of Paracetamol and Orphenadrine Citrate in dosage formulations and in human Serum	1.5 mL/min	1.7 for PAR.	96.96 - 100.02 for PARat various added concentration	101.7 - 102.2 for PAR at various added concetration	(6-1000) ng/mL for PAR.	acetonitrile: water (50:50), adjusting pH to 2.6 with phosphoric acid, C_{18} column (10 µm,250 mm x 0.46 mm), 215 nm .	[46]
Simultaneous estimation of Paracet-amol and Lornoxicam in combined tablet dosage form and its dissolution assessment	1 mL/min	4.325 for PAR.	100.11 for PAR.	0.26 for PAR.	LOD 0.021 ng/mL for PAR	ethyl acetate: methanol: water (2.5: 70:28.5 v/v), pH adjusted to 4.0 with acetic acid. C_{18} (250 mm x4.6 mm ,4 µm), 234 nm	[47]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous estimation of Paracetamol and Ibuprofen in tablets	0.8 mL/min	4.45 for PAR.	98.90 for PAR.	< 2% for PAR.	(20-80) µg/mL ,LOD (2ng/mL) for PAR	acetonitrile: phosphate buffer (60:40, v/v, pH 7.0), C18 (150 mm x 4.6 mm, 5 µm), 260 nm.	[48]
Simultaneous estimation of Pyridoxine, Thiamine, Paracetamol and Ibuprofen in tablets	1 mL/min	7.40 for PAR.	98.6 for PAR.	< 2% for PAR.	(0.25-1.00) mg/mL for PAR.	acetonitrile : phosphate buffer (v/v)pH (7.0),C_8 (250 mm x 4.6 mm, 5 µm),220 nm	[49]
Simultaneous estimation of paracetamol and tramadol hydrochloride in a commercial tablet.	1 mL/min	3.9 for PAR.	99.23 for PAR	0.16 for PAR.	(20.8-39.0) µg/mL for PAR	methanol: buffer: water (adjusted pH 3.4 with ortho-phosphoric acid) (40:60, v/v), C_{18} (250 mm x 4.6 mm , 5 µm) , 228 nm.	[50]
Determination of Psuedoephedrine hydrochloride, Cetirizine dihydrochloride and Paracetamol Uncoated tablet.	1 mL/min	3.569 for PAR.	100.61 for PAR.	0.2 for PAR.	(20-160) mcg/mL for PAR.	buffer: acetonitrile (85:15) ,C_8 (250 mm x 4.6 mm),215 nm .	[51]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous determination of Aceclofenac, paracetamol and Tizanidine in pharmaceutical preparations	0.7 mL/min	3.840 for PAR.	100.95 - 99.99 for PAR at various added concentration	0.52 for PAR	(500-1500) µg/mL, LOD 0.3 µg/mL for PAR.	phosphate buffer pH 7.0: acetonitrile (40:60 v/v), C_{18} (250 mm x 4.6 mm , 5 µm) , 230 nm .	[52]
Quantitative analysis of mitragynine, Codeine, Caffeine, Chlorpheniramine and Phenylephrine in a kratom (Mitragyna speciosa Korth.) Cocktail	1 mL/min to separate CAF. 1.5 mL/min to separate CP	2.340 for CAF. 3.159 for CP.	95.83 for CAF. 100.56 for CP.	0.559 for CAF. 0.581 for CP.	(0.4-500) mg/mL ,LOD 0.005 mg/mL for CPM. (0.01-200) mg/mL ,LOD 0.200 mg/mL for CAF.	(0.01 M) KH_2PO_4 : methanol: acetonitrile : isopropanol (74:8:9:9, v/v/v/v) to separate caffeine, 10 mM phosphate buffer at pH 3.0: acetonitrile (96:4, v/v) to separate CP	[53]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous determination of Paracetamol, Acetyl Salicylic Acid, Mefenamic Acid and Cetirizine Dihydrochloride in the pharmaceutical dosage form	1 mL/min	2.92 for PAR	99.4 - 99.5 for PAR.	0.86 for PAR	(30-100) µg/mL ,LOD 0.39 µg/mL for PAR.	20 mmol Na_2HPO_4 pH=6.5: acetonitrile (60:40 v/v), C_{18} (250 x 4.6 mm, 5 µm), 220 nm.	[54]
Simultaneous determination of paracetamol and mefenamic acid in tablet dosage form by high performance liquid chromatography	1 mL/min	2.88 for PAR.	98.38 - 100.73 for PAR at various added concentration	0.875 - 0.227 for PAR at various added concentration	(36-180) µg/mL for PAR.	buffer (0.02M KH_2PO_4) (75:25), pH 7.1 adjusted with 0.1N NaOH, Column ODS C_{18} (250 x 4.6 mm, 5 µm), 275 nm.	[55]
Rapid and Simultaneous determination of Acetylsalicylic Acid, Paracetamol, and their degradation and toxic impurity products by HPLC in pharmaceutical dosage forms	1.8 mL/min	2.66 for PAR.	100.00 for PAR.	0.96 for PAR.	(0.5-4.0) µg/mL ,LOD 0.036 µg/mL for PAR	methanol: water (35:65; v/v) adjusted to pH 3.1 with 10% orthophosphoric acid, Column C_{18} (300mm x 3.9mm), 235 nm.	[56]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Development and validation of RP-HPLC method for simultaneous estimation of Nimesulide ,Phenylephrine hydrochloride,Chlophenir amine maleate and Caffeine anhydrous in pharmaceutical dosage form.	1 mL/min	17.15 for CPM and 4.55 for CAF	100.30 for CPM and 99.97 for CAF	Less than 2%	(16-32) µg/mL for CPM and (30-180) µg/mL for CAF , LOD 1.14 µg/mL for CPM and 4.55 µg/mL for CAF	methanol : buffer solution(KH_2PO_4 ,TEA HCl,H_3PO_4, $C_{12}H_{25}NaO_4S$) (55:45)pH=5.5 , Column C_{18}(4.6 mm x25cm),214 nm	[57]
HPLC assay and stability of tablets containing Pracetamol and Caffeine.	1 mL/min	3.366 for PAR and 5.443 for CAF.	99.57 for PAR and 99.87 for CAF.	0.80 for PAR and 0.70 for CAF.	(31.25-250) µg/mL for PAR and (4.06-32.50) µg/mL for CAF	1 mM phosphate buffer (pH=7.0): methanol (65:35 v/v) C_8 column (250 mm x 4.6 mm, 5µm), 230 nm.	[58]
Simultaneous determination and validation of Paracetamol and Codeine phosphate in pharamaceutical preparation by RP-HPLC	1 mL/min	5.216 for PAR	99.88 for PAR	0.39 for PAR	(100-1000) µg/mLfor PAR.LOD 1 µg/mL for PAR.	acetonitrile: 1ml TEA and buffer solution (pH=2.5), C_{18} (250 mm x 4.6 mm,5µm) ,210 nm	[59]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Development and validation of RP-HPLC of simultaneous estimation of Tolperisone hydrochloride and Acetaminophen in tablet dosage form.	0.7 mL/min	3.29 for PAR.	99.88-100.96 for PAR at various concentration	0.3974 for PAR	(2-10) µg/mL, LOD 0.02 µg/mL for PAR.	acetonitrile: water (40:60v/v) (pH=3), C_{18} column (250 mm X4.6mm), 258 nm.	[60]
Simultaneous determination of Pracetamol and Aspirin in tablet.	1 mL/min	3.052 for PAR	99.20 for PAR	0.41 for PAR	(2-64) µg/mL for PAR.	acetonitrile: phosphate buffer pH=7.0 (60:40 v/v), C_8 (125mm x 4.6 mm, 5µm), 230 nm.	[61]
Simultaneous determination of Paracetamol and its main degradation product in generic Paracetamol tablets using reverse-phase HPLC.	1 mL/min	5.987 for PAR.	103.61 for PAR.	1.81 for PAR.	(2.5-20) µg/mL for PAR. LOD 0.006 µg/mL for PAR.	methanol: 0.1 M phosphate buffer pH 5.0(30:70) ,C_{18} column Inertsil (250 mm x 4.6 mm , 5µm), 243 nm.	[62]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Quantitation of Acetaminophen in pharmaceutical formulations using high-performance liquid chromatography	1.7 mL/min	2.580	99.3-99.2	0.99	(10-30) µg/mL	methanol: water (330:660, v/v) pH= 3.0 adjusted by 10% H_3PO_4, C_{18} column (30 cm x 3.9 mm, 10 µm) , 193.3 nm.	[63]
Simultaneous determination of Paracetamol, Caffeine, Guaifenesin and Preservatives in syrups by Micellar LC.	1 mL/min	2.23 for PAR and 5.32 for CAF.	99.87 for PAR and 99.4 for CAF	1.12 for PAR and 0.36 for CAF.	(37-46) µg/mL for PAR. and (6-130) µg/mL for CAF.LOD 0.28 µg/mL for PAR and 2.65 µg/mL for CAF.	1-butanol:water (1:99, v/v), containing 0.04M sodium dodecyl sulfate and 0.1% trichloroacetic acid., Kromasil C_{18} column (150 mm x4.6 mm, 5µm) ,260 nm.	[64]
A Simple and Sensitive HPLC Method for simultaneous analysis of Nabumetone and Paracetamol in pharmaceutical formulations	1 mL/min	2.653 for PAR.	99.67-101.32 for PAR at various concentrations.	0.524-0.872 for PAR at various concentrations.	(5-25) µg/mLfor PAR.	cetonitrile:0.05% aqueous acetic acid (70:30v/v), Hypersil C_{18} column (250 mm × 4.6 mm), 238 nm.	[65]

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous HPLC determination of Methocarbamol, Paracetamol and Diclofenac Sodium	1 mL/min	4.15 for PAR.	99.76 µg/ml for PAR.	1.524 for PAR.	(3.25-29.25) µg/mL for PAR.	methanol: water: glacial acetic acid ,(400:600: 05) , HiQ Sil C_{18} column (4.6 mm ID × 250 mm), 275 nm.	[66]
Method development and validation for the simultaneous determination of Paracetamol and Tramadol in sold dosage form by RP-HPLC	1 mL/min	3.34 for PAR.	100.20 for PAR.	0.090 for PAR.	(1.0-12.0) µg/mL, LOD 0.12 µg/mL for PAR	acetonitrile - 0.26 % triethylamine buffer (pH 7.3) in (45:55 % v/v) , C_{18} column (4.6 mm ID×250 mm) , 264 nm.	[67]

Results for the present work

Compound	Flow rate	t_R min	Recovery%	R.S.D%	Linearity and/or LOD	Conditions	Ref.
Simultaneous determination and validation of Chlorpheniramine Maleate, Acetaminophen, Phenylpropanolamine Hydrochloride and Caffeine in Solid Pharmaceutics by using reveres phase high performance liquid Chromatography.	1mL /min (in 0-5 min) to 1.7 mL/min (after 5 min) till 8 min.	3.423 for CPM, 5.627 for PAR, 6.052 for PPA and 7.690 for CAF.	(101.650-101.640) for CPM, (100.305-99.484) for PAR, (100.840-100.751) for PPA and (98.075-100.461) for CAF.	(0.200-0.252) for CPM, (0.207-0.065) for PAR, (0.406-0.371) for PPA (0.020-0.020) for CAF at various concentration.	(1-50) µg/mL, LOD (0.516) , LOQ (1.72)for CPM, (1-500), µg/mL , LOD (0.424) ,LOQ(1.416) for PAR, (1-200), µg/mL, LOD (0.465), LOQ (1.552) for PPA and (1-150) µg/mL, LOD (0.683), LOQ (2.277) for CAF.	acetonitrile- water - methanol- orthophosphoric acid (1M)- triethylamine= hexansulfonic acid (14mmole/L) as ion pair and with the volume ratio (v/v) of (15:75:10:0.5:0.3: 4) ;respectively ,adjusting pH to 2.8 by diluted orthophosphoric acid. ODS2-C18 (250 mm x 4.6mm, 5µm particle) analytical column at 25 ^{o}C. 220 nm.	**Present work**

Chapter two

Experimental part

(2-1) Apparatus:

1. Double beam UV-Visible – spectrophotometer -1800, Shimadzu, (Japan). Equipped with quartz cell (1cm).
2. High-Performance Liquid Chromatography (HPLC), UFLC - Shimadzu, CBM 20A, (Japan) , Equipped with ODS2-C_{18} (250 mm x 4.6mm, 5µm particle) analytical column, UV-Visible detector and Column oven CTO-20A (4- 85) ^{o}C, Shimadzu (Japan) , manual injection.
3. Digital Microbalance, Sartorius (Maximum weight 2.1 g) - CPA2P, (Germany).
4. Digital Balance, Denver –TP-214, (Germany).
5. FT-IR, Bruker, TENSOR 27, (Germany).
6. pH-meter, Hanna-pH211, (Romania.)
7. Ultrasonic cleaner, KQ200E, (Chain).

(2-2) Drugs and chemical materials

The drugs and chemical materials were shown in table (2-1).

Table (2-1): The drugs and chemical materials used in this study.

Name	Chemical structure	Purity	Manufactured by companies
Methanol ,HPLC grade	CH_3OH	99.85%	England
Acetonitrile, HPLC grade	CH_3CN	99.7%	Sharlu
Potassium dihydrogen phosphate	KH_2PO_4	98%	Thomas Baker
Standard Acetaminophen	$C_8H_9NO_2$	<99.5%	S.D.I
Standard Caffeine	$C_8H_{10}N_4O_2$	<99.5%	S.D.I
Standard Chlorpheniramine maleat	$C_{20}H_{23}ClN_2O_4$	<99.5%	S.D.I
Phenylpropanolamine hydrochloride	$C_9H_{14}ClNO$	<99.5%	S.D.I
Orthophosphoric acid	H_3PO_4	85.5%	Himedia
Hexansulfonic acid sodium salt monohydrate	$CH_3(CH_2)_5SO_3Na.H_2O$	< 98%	Fluka AG
Triethylamine	$(CH_2)_3N$	< 98%	Labosi
Glacial acetic acid	CH_3COOH	< 98%	Sharlu

(2-3) Preparation of standard stock solutions

Standard stock solutions of CPM, PAR, PPA and CAF were prepared by accurately weighing 50mg from each drug individually and qualitatively transferred into a 50 ml volumetric flask and complete the volume with mobile phase. The mixture was sonicated for 15 min or until the reference standard dissolved completely. From the standard stock solutions, serial dilutions in mobile phase were made to prepare standard curves.

(2-4) Preparation of sample solutions.

Twenty tablets (Dolo cold, India), each containing 2 mg CPM, 500 mg PAR, 12.5 mg PPA and 25 mg CAF are weighed and powdered finely. A quantity of powdered which is equivalent to one tablet (contains 2 mg CPM, 500 mg PAR, 12.5 mg PPA and 25 mg CAF) was weighed accurately and transferred into 200 mL calibrated volumetric flask. About 50 mL of diluent was added and ultrasonicated for 15 min; finally the volume adjusted to the mark. The resulting solution obtained was then filtered, through 0.45 µm filter paper for removal of particulate matter. 5 mL of the filtrate was diluted to 25 mL in the volumetric flask with the diluent for analysis, same procedure for the rest of tablets under assay (Panadol Extra and Panadol cold and flu).

(2-5)Preparation of Hexansulfonic acid sodium salt monohydrate (HAS) (14mmol L^{-1}).

Prepared by dissolving 0.0722 gm of $CH_3(CH_2)_5SO_3Na.H_2O$ in 10 mL double distilled water and the volume completed to (25 mL) with double distilled water in a volumetric flask .

(2-6) Preparation of orthophosphoric acid (1M)

A 0.684 mL of concentrated orthophosphoric acid transfer to 10 mL volumetric flask, and diluted with double distilled water to the mark.

(2-7) Preparation of potassium dihydrogen phosphate buffer (1% w/v).

Accurately weighed 1.00 gm potassium dihydrogen phosphates were quantitatively transferred to 100 mL volumetric and dissolved with double distilled water. The obtained solution was completed to the mark with double distilled water. The pH was adjusted to 2.8 by addition a drops of diluted orthophosphoric acid.

(2-8) Wavelength selection

The individual drugs substance solutions at concentration of 100 µg/mL in diluent were scanned by UV-Visible spectrophotometer in the range of (200-400) nm. From the overlain UV spectra, suitable wavelength considered for monitoring the drugs were 220 nm on basis of higher response [68].

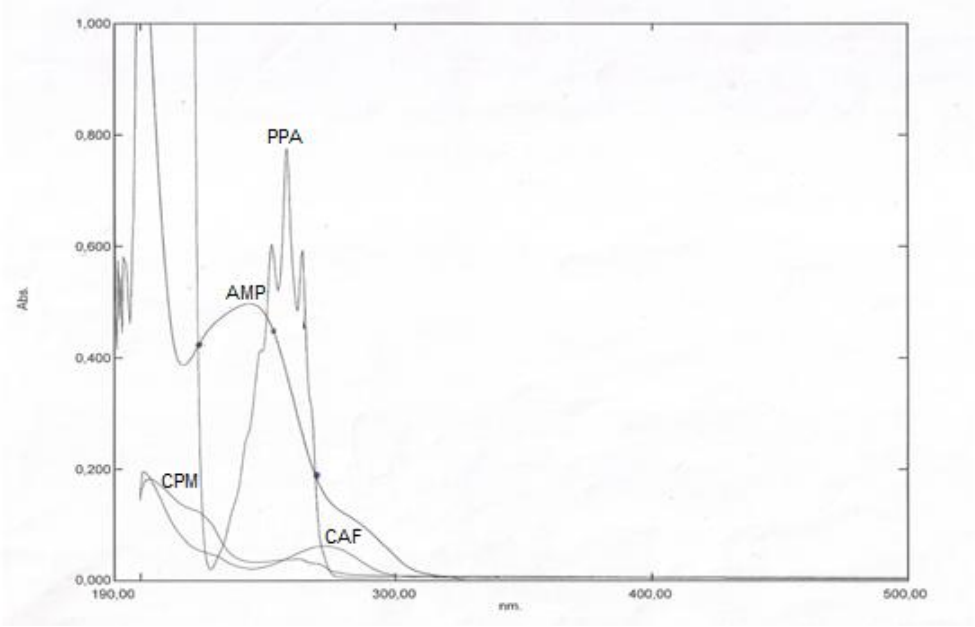


Fig.(2-1): Overlain spectrum for standard solution of CPM, AMP, PPA and CAF.

(2-9) FT-IR spectra of drugs:-

FT-IR spectra were recorded for the compounds. The spectra were compared with standards spectra in order to identify these compounds.

(2-10) Chromatographic conditions of isocratic elution system

HPLC analysis was performed by isocratic elution .The flow rate was changed from 1 mL/min (in 0-5 min) to 1.7 mL/min (after 5 min) till 8 min. The mobile phase composition was acetonitrile- water - methanol- orthophosphoric acid (1M)- triethylamine (TEA)- Hexansulfonic acid sodium salt monohydrate (HSA) (14mmole L^{-1}) as ion pair [69,70]

(15:75:10:0.5:0.3:4, v/v/v/v/v/v), respectively, adjusting pH to 2.8 by diluted orthophosphoric acid . All solvents were filtered through a 0.45 µm filter paper and degassed in an ultrasonic bath. Volumes of 20 µL of prepared solutions and samples were injected into the column. Quantification was effected by measuring at 220 nm. The chromatographic run time was less than 8 min and the column void volume was 2.200 min. Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k')[71], the resolution (R_s), the selectivity (α),number of theoretical plate (N) and peak asymmetry (T) [72].

(2-11) Calibration curve

Standard solutions containing CPM (1-50) µg/mL, PAR (1-500) µg/mL, PPA (1-200) µg/mL and CAF (1-150) µg/mL were prepared in the mobile phase. Triplicate 20 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The concentrations of each compound were subjected to regression analysis to calculate the calibration equation and correlation coefficients.

(2-12) Optimization of HPLC method

A single and high resolution RP-HPLC method has been developed for the quantification of CPM, PAR, PPA and CAF in pharmaceutical formulations. Initial stages of method development, trials were performed with a mixture of aqueous acetonitrile, methanol, and disodium hydrogen phosphate dihydrate buffer in various proportions (pH 2-4.5 adjusted with

diluted orthophosphoric acid) with C_{18} (250mm x 0.45 mm, 5 µm) column but separation was not achieved. Finally the separation was achieved by using a mixture of acetonitrile- water- methanol- orthophosphoric acid (1M)- TEA- HSA (14mmole L^{-1}) as ion pair with the volume ratio (v/v) of (15:75:10: 0.5:0.3:4) , respectively, adjusting pH to 2.8 by diluted orthophosphoric acid, at flow rate from 1 mL/min (in 0-5 min) to 1.7 mL/min (after 5 min) with isocratic program and gave acceptable retention time of (3.423, 5.627 , 6.052 and 7.690) min with number of plates (N) (1446.535, 3166.313 , 2893.962 and 3784.711) for CP, PAR, PPA and CAF ,respectively, and good resolution (5.800 between CPM and PAR), (1.000 between AMP and PPA) and (3.449 between PPA and CAF and Tailing factor (not more than 1.0) were within the limit.

(2-13) Validation of the method

Validation of the optimized HPLC method was carried out with respect to the following parameters: -

(2-13-1) Linearity and range

Linearity of the method was studied by injecting the concentrations of the drugs prepared in the mobile phase in the range of (1-50) µg/mL for CPM, (1-500) µg/mL for PAR, (1-200) µg/mL for PPA and (1-150) µg/mL for CAF; in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

(2-13-2) Precision

The precision of the method was verified by repeatability [73,74,75]. Repeatability studies were performed by analysis of three different concentrations (2, 30, 50) µg/mL for CPM, (20, 300, 500) µg/mL for PAR, (5, 30, 100) µg/mL for PPA and (20, 50, 150) µg/mL for CAF three times on the same day.

(2-13-3) Accuracy

The accuracy of a method is the closeness of the measured value to the true value for the sample. For pharmaceutical studies, the most widely used approach is the recovery study [76,77], which is performed by spiking analyte in blank matrices. It was tested in the same linearity assay for the four main components.

(2-13-4) Specificity

The specificity of the method was assessed by comparing chromatogram obtained from drug standard with that from marketed tablets solutions [31].

(2-13-5) System suitability

System suitability is defined as, the checking of a system, before or during analysis of unknowns, to ensure system performance. A data from three injection of system precision were utilized for calculating system suitability parameter like R.S.D%, Tailing factor, capacity factor, selectivity, resolution and theoretical plates [41]. ODS-2 inertsil C_{18}

column (250 mm x 4.6mm, 5µm) was used as stationary phase. The mobile phase consists of acetonitrile- water - methanol- orthophosphoric acid (1M)- (TEA)- HSA (14mmole L^{-1}) as ion pair (15: 75: 10: 0.5:0.3:4, v/v/v/v/v/v) , respectively, adjusting pH to 2.8 by diluted orthophosphoric acid. 220 nm was the detection wavelength. Flow rate was 1 mL/min (in 0-5 min) to 1.7 mL/min (after 5 min). Column runs for 8 min at temperature of 25^{o}C.

(2-13-6) Limits of detection and Limit of quantitation

Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) [78] and Limit of Quantitation (LOQ). LOD = 3 SD/S and LOQ = 10 SD /S, where S.D. is the standard deviation of y-intercept and S is the slope of the line [79].

(2-14) Analysis of a marketed formulation

To determine the content of CPM, PAR, PPA and CAF in commercial drugs illustrated below:-

Table (2-2): Marketed formulation under assay.

Brand name	Manufactured	Label claim
Dolo Cold	MICRO LASS ,India	2 mg CPM, 500 mg AMP, 12.5 mg PPA and 25 mg CAF.
Panadol Cold & Flu	GlaxoSmithKline, Australia	500gm AMP, 30 mg P and 2 mg CPM.
Panadol Extra	GlaxoSmithKline, Ireland	500 mg AMP and 65mg CAF

A 20µL volume of sample solution was injected into HPLC system, three times, under the conditions described above. The peak areas were measured at 220 nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

(2-15) Effect of pH

The effect of changing pH of the mobile phase on the selectivity and retention times of the test solutes was investigated using mobile phases of pH ranging from (2.8 - 4.7) under optimum condition.

(2-16) Effect of variation of flow rate

A study was conducted to determine the effect of variation in flow rate under optimum condition. Standard solution prepared as per the test method was injected into the HPLC system using flow rates (1.0, 1.2, 1.4 and 1.7) mL/min.

(2-17) Effect of temperature

The effects of column temperature on resolution in reversed-phase high performance liquid chromatography (RP-HPLC) in the range of (25 – 40) ^{o}C were investigated under optimum condition.

(2-18) Effect of volume injection

The effects of variable volume injection (5, 10, 15 and 20) µl were studied under optimum condition.

Chapter three

Results and Discussions

(3-1) Study of FT-IR spectra [80]*:*

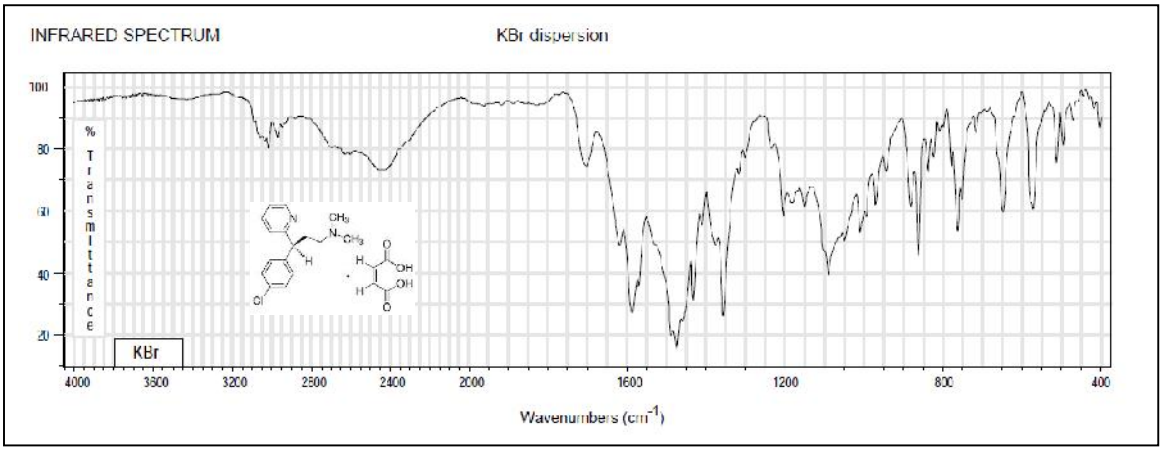


Fig.(3-1):FT-IR spectra for standard Chlorpheniramine maleate (Sigma –Aldrich)

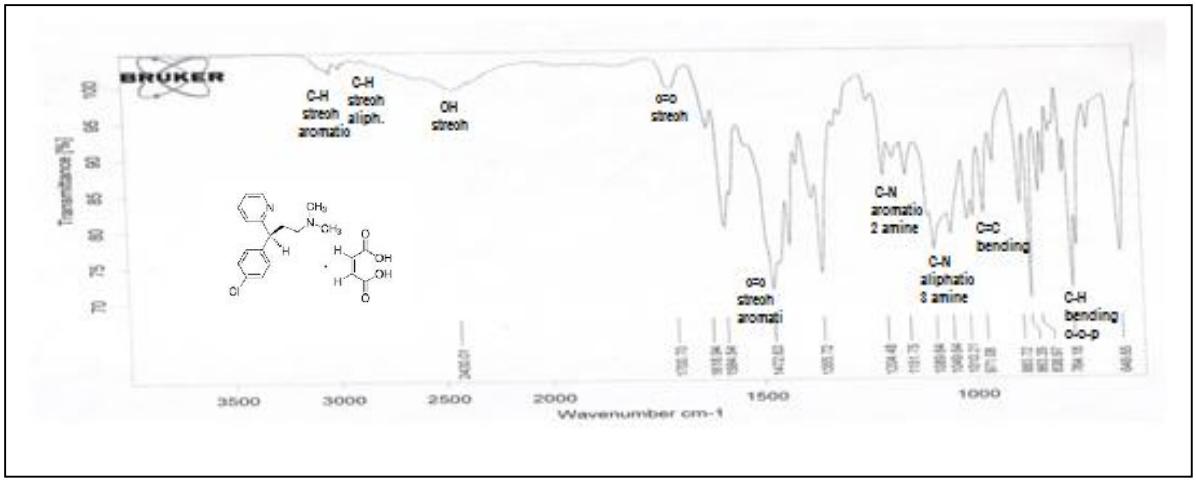


Fig.(3-2): FT-IR spectra for standard Chlorpheniramine maleate (S.D.I)

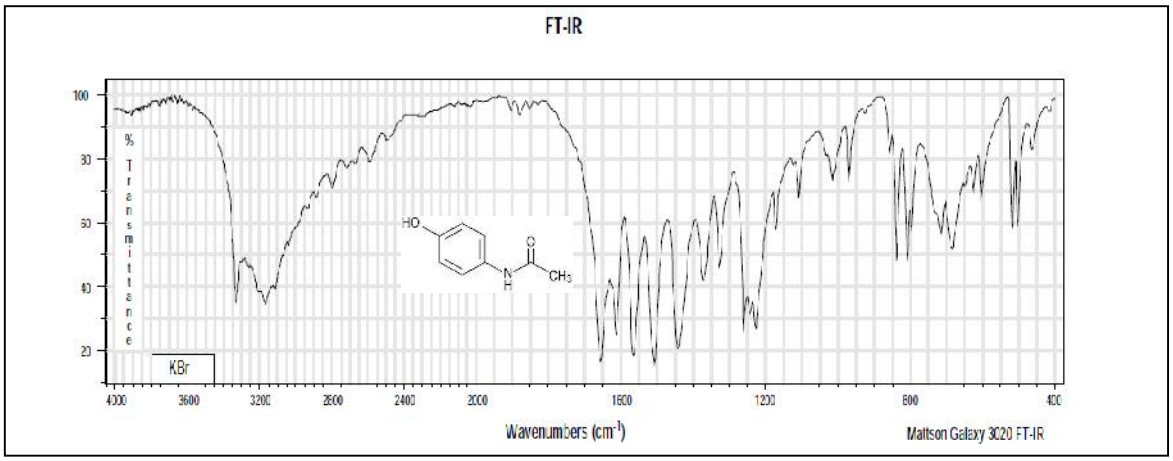


Fig.(3-3)FT-IR spectra for standard Paracetamol (Sigma–Aldrich)

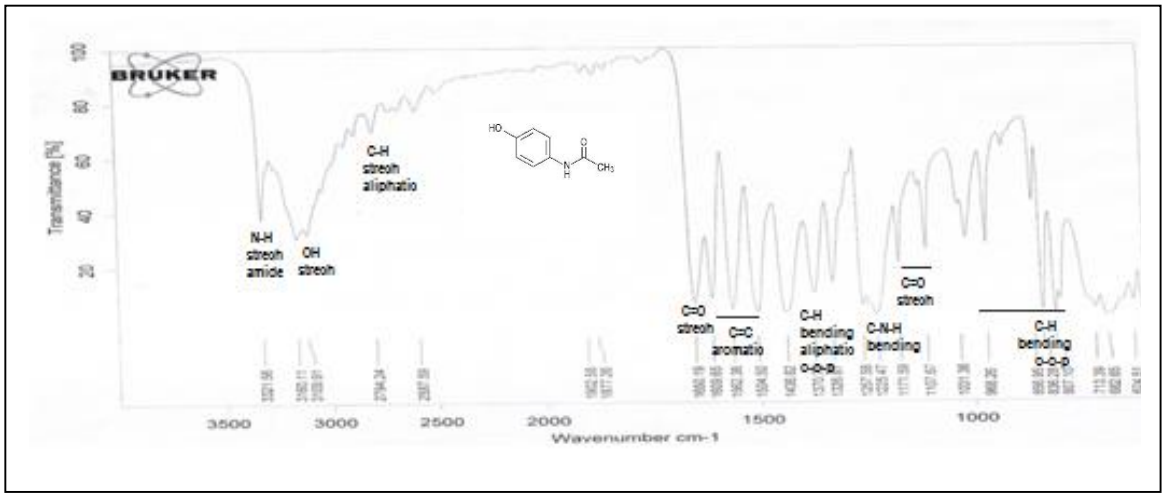


Fig.(3-4): FT-IR spectra for standard Paracetamol (S.D.I)

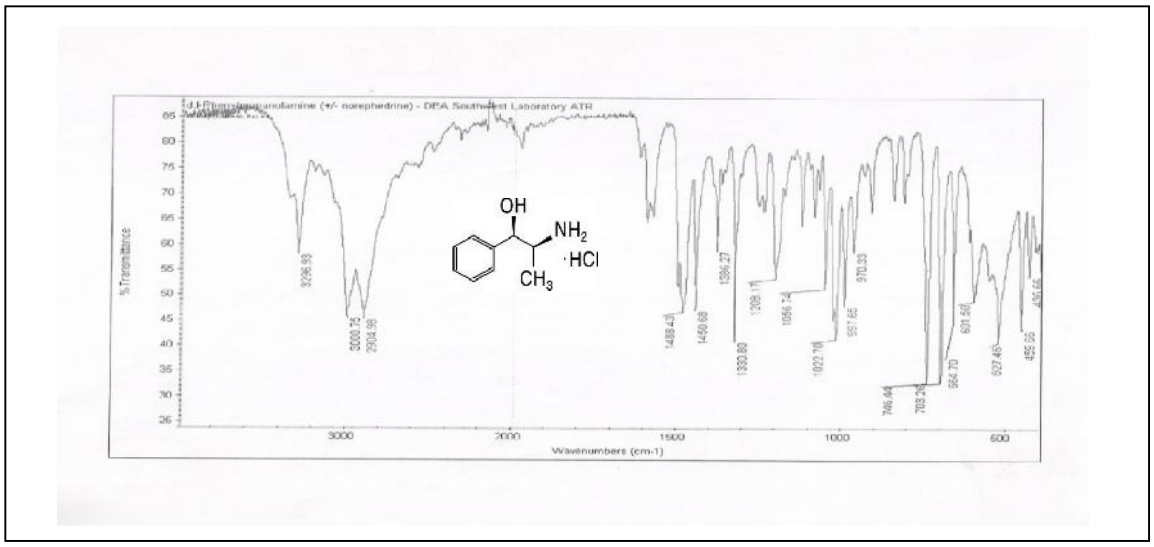


Fig.(3-5): FT-IR spectra for standard Phenylpropanolamine hydrochloride (DEA Southwest Lab.)

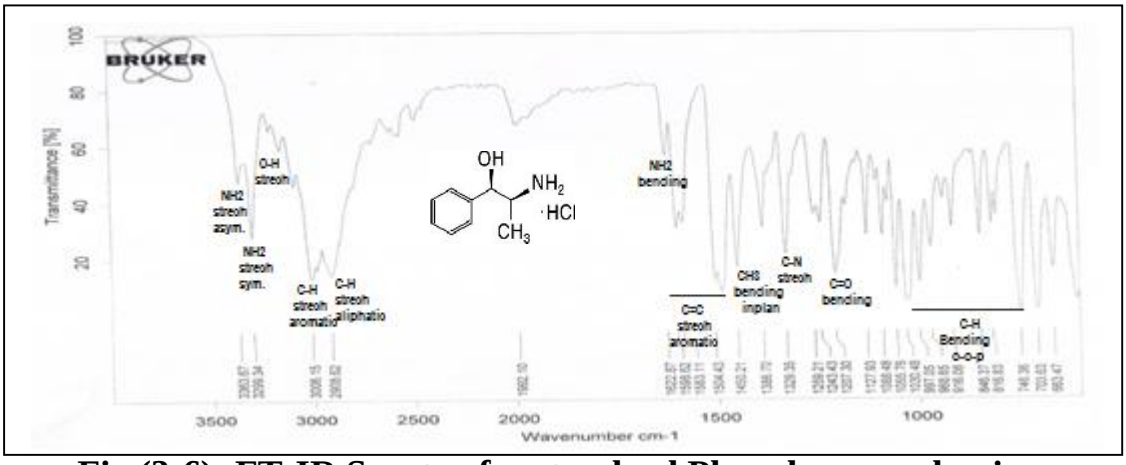


Fig.(3-6): FT-IR Spectra for standard Phenylpropanolamine hydrochloride (S.D.I)

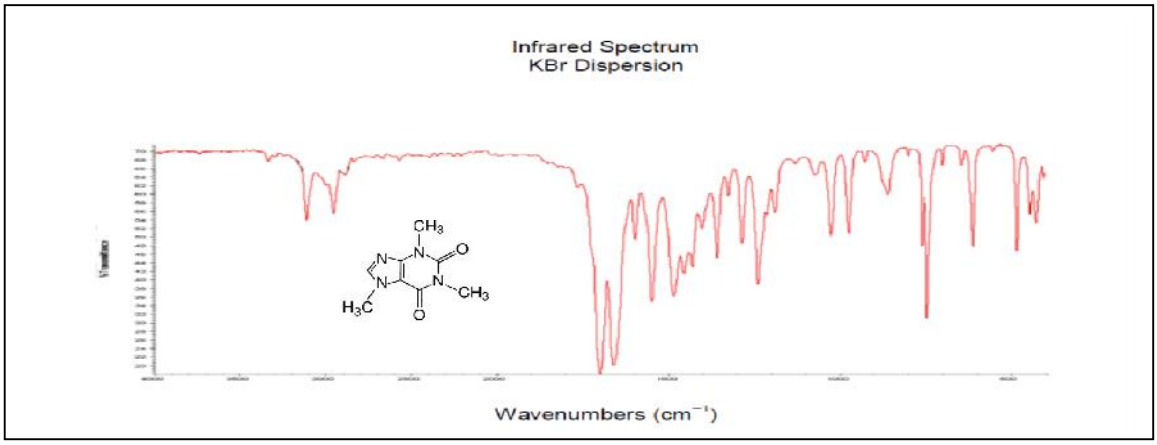


Fig.(3-7): FT-IR spectra for standard Caffeine (Sigma –Aldrich)

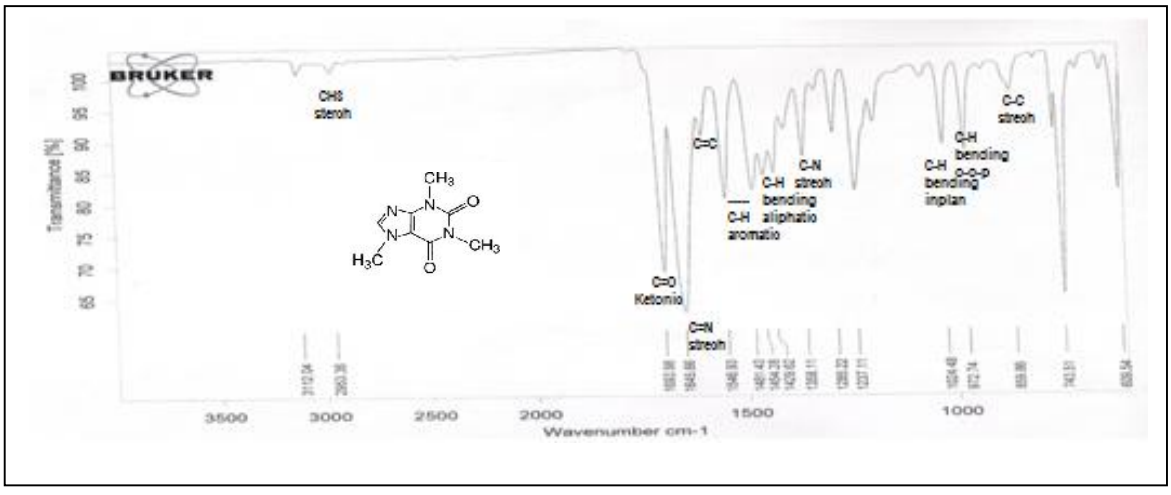


Fig.(3-8): FT-IR spectra for standard Caffeine (S.D.I)

(3-2)Validation of the method

Validation of the optimized HPLC method was carried out with respect to the following parameters

(3-2-1) Calibration curve and linearity study

CPM, PAR, PPA and CAF showed good correlation coefficient in concentration range of (1-50) µg/ml, (1- 500) µg/ml, (1-200) µg/ml and (1-150) µg/ml, respectively .Above these rang deviation from Lambert low appeared. The detector response over wide range of concentrations of analyte were plotted to obtain the calibration curve [figures (3-1), (3-2), (3-3) and (3-4)].

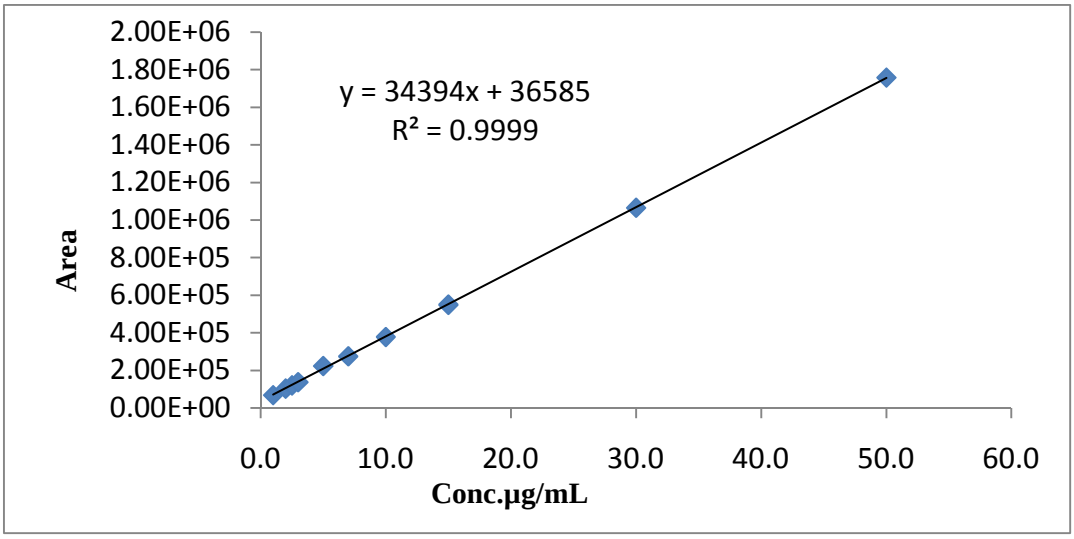


Fig.(3-9): Calibration curve of standard solution of CPM .

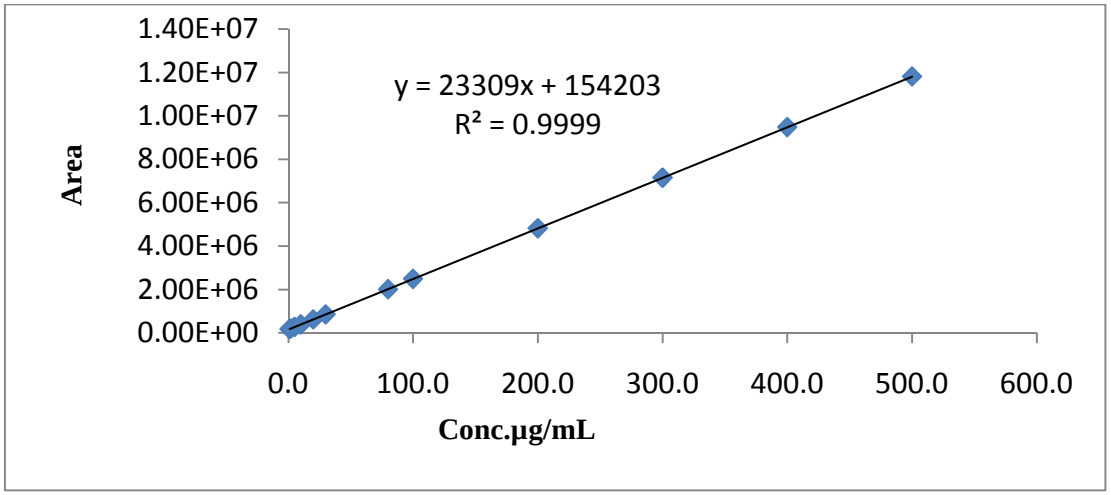


Fig.(3-10): Calibration curve of standard solution of PAR.

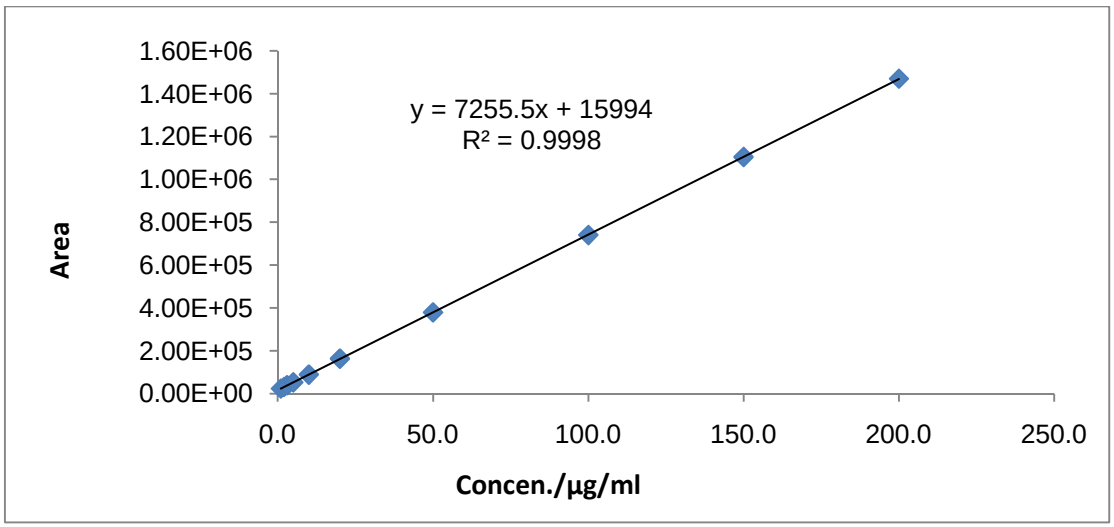


Fig.(3-11): Calibration curve of standard solution of PPA.

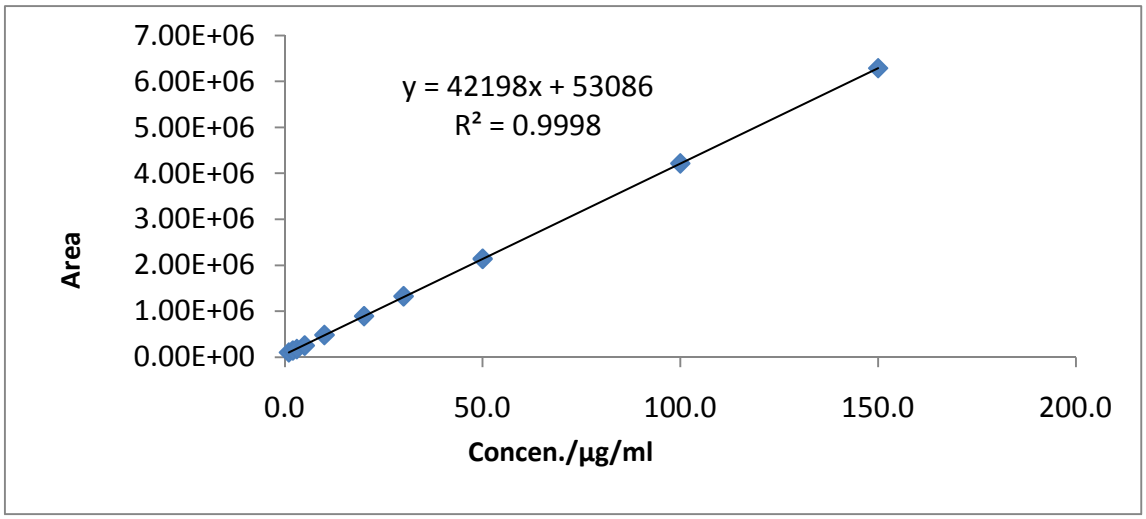


Fig.(3-12): Calibration curve of standard solution of CAF.

The square of the correlation coefficients and equations for the curves are shown in table (3-1).

Table (3-1) linearity and regression characteristics of standard drugs.

Parameters	CPM	PAR	PPA	CAF
Linearity range µg/mL	1-50	1-500	1-200	1-150
Regression equation	Y = 34394x + 36585	Y = 23309x +154203	Y = 7255.5x + 15994	Y = 42198x + 53086
Correlation coefficient(r2)	R^2 = 0.9999	R^2 = 0.9999	R^2 = 0.9998	R^2 = 0.9998

All four (R^2) values are greater than 0.9997. From these results it is acceptable to use a single point calibration in analysis of actual samples.

(3-2-2) Precision

Precision was evaluated by carrying out three different sample preparations for all individual and combination dosage forms. Results were shown in table (3-2).

Table (3-2): Precision for standard drugs.

Drugs	(µg/mL)	R.S.D% (n=3)
CPM	2.000	0.2000
	10.000	0.027
	50.000	0.252
PAR	20.000	0.207
	300.000	0.023
	500.000	0.065
PPA	5.000	0.406
	30.000	0.857
	100.000	0.371
CAF	20.000	0.020
	50.000	0.126
	150.000	0.020

Percentage relative standard deviation (R.S.D %) was found to be less than 1, and E% less than 6 which proves that the developed method is precise and reproducible [81].

(3-2-3) Accuracy

The accuracy of method was confirmed by studying recovery at three different concentrations for all samples, by replicate analysis (n=3). Samples of known concentration (reference standard solutions) were analyzed and the measured values, from the respective area counts, were compared with the true values. The results obtained from the determination of accuracy, expressed as percentage recovery, are summarized in table (3-3).

Table (3-3) Percentage recovery data for standard drugs

Drugs	Amount added (µg/mL)	Amount found (µg/mL)	Recovery [%] (n=3)	E% (n=3)
CPM	2.000	2.033	101.650	1.650
	10.000	10.583	105.830	5.830
	50.000	50.820	101.640	1.640
PAR	20.000	20.061	100.305	0.305
	300.000	300.301	100.100	0.100
	500.000	497.419	99.484	-0.516
PPA	5.000	5.042	100.840	0.840
	30.000	30.240	100.800	0.800
	100.000	100.751	100.751	0.751
CAF	20.000	19.615	98.075	-1.925
	50.000	50.749	101.498	1.498
	150.000	150.691	100.461	0.461

From these results the method enables accurate quantitative estimation of CPM, PAR, PPA and CAF, because all the results were within acceptable limit [3].

(3-2-4) Specificity

The retention time of the standard drugs and the drugs solutions were illustrated in [figures (3-5), (3-6), (3-7) and (3-8)] and table (3-4).

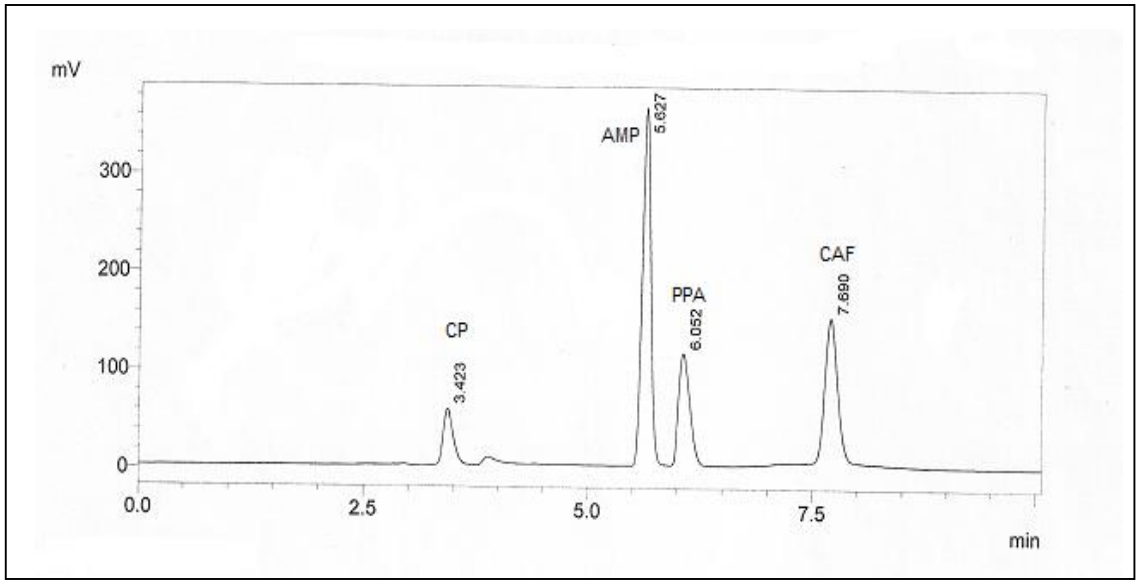


Fig.(3-13): Chromatogram of standred drugs solutions of CPM, PAR, PPA and CAF.

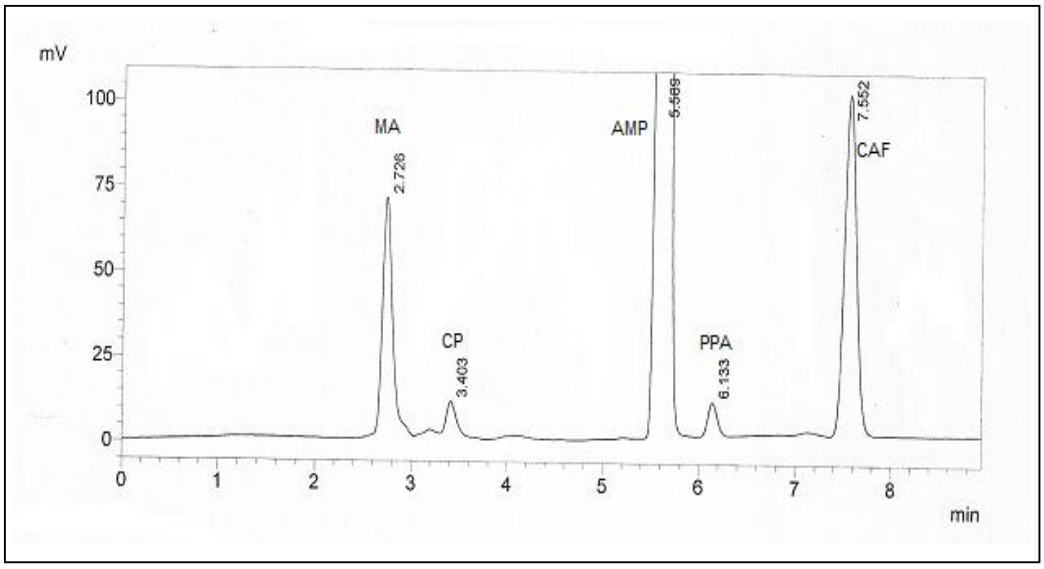


Fig. (3-14) Chromatogram of drug(1) (contain CPM, PAR, PPA and CAF)

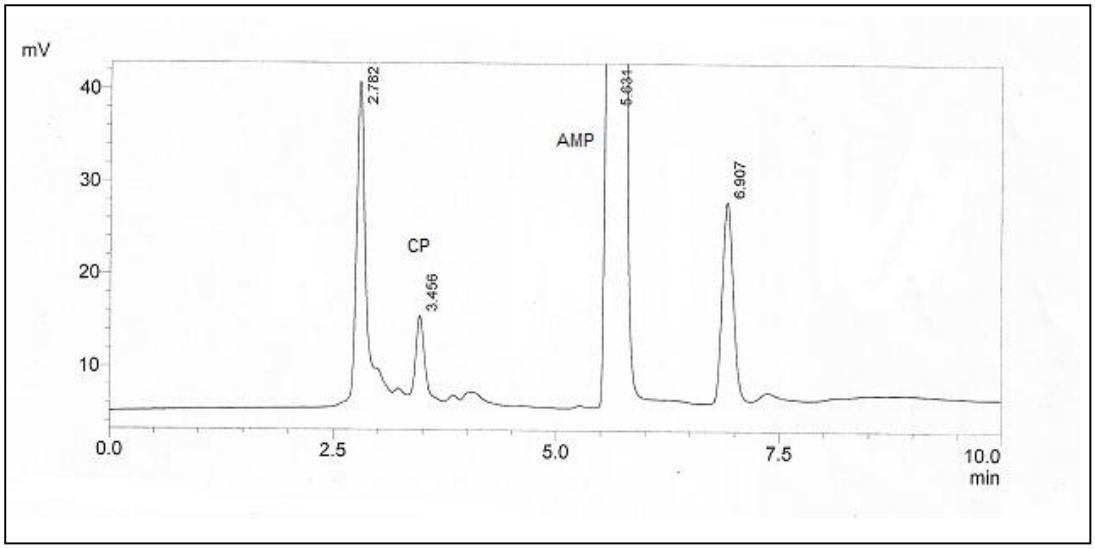


Fig. (3-15): Chromatogram of drug (2) (contain CPM and PAR)

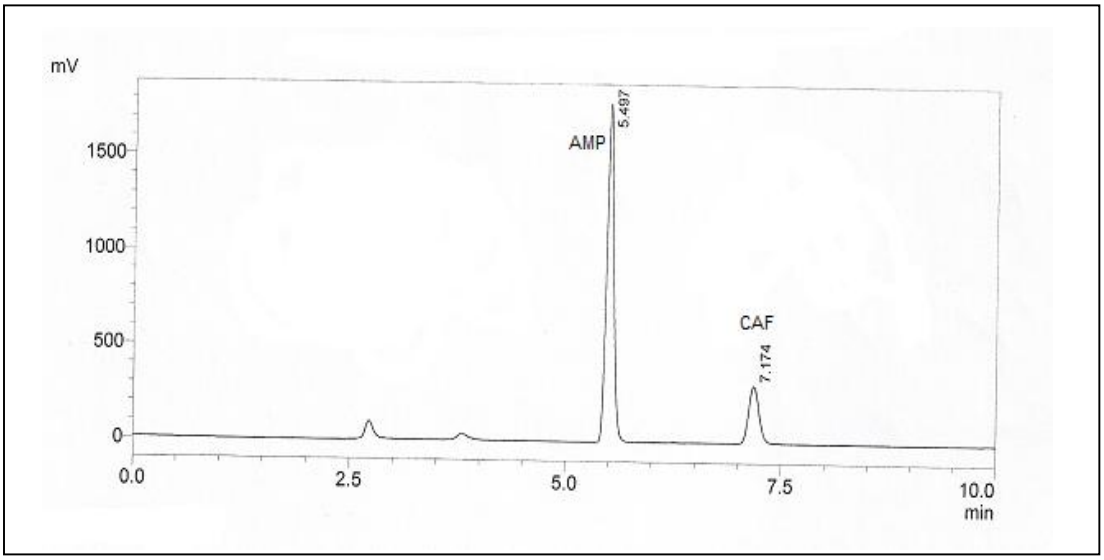


Fig. (3-16): Chromatogram of drug (3)(contain PAR and CAF)

Table (3-4): Retention time of three assayed drugs and standard drugs solutions

Drugs	t_R			
	CP	**PAR**	**PPA**	**CAF**
Standard drugs solution	3.423	5.627	6.052	7.690
Dolo cold	3.403	5.589	6.133	7.552
Panadol cold &Flow	3.403	5.631	------	------
Panadol extra	------	5.497	------	7.174

Result in table (3-4) showed that the retention time of the standard drugs and the drugs solutions were very colse , almost same , so the method was specific .

(3-2-5) Limit of detection (LOD) and Limit of quantitation (LOQ)

The results of limit of detection and limit of quantification were illustrated in table (3-5).

Table (3-5): Limit of detection and Limit of Quantification

Drugs	(S.D %) (n=3)	Slope	LOD µg/mL	LOQ µg/mL
CP	5916.2	34394.0	0.516	1.720
AMP	3300.103	23309	0.425	1.416
PPA	1126.085	7255.5	0.466	1.552
CAF	9608.63	42198	0.683	2.277

The values indicate that the method is sensitive [10].

(3-2-6) System suitability parameters

For system suitability parameters, three replicate injections of mixed standard solution were injected and parameters such as the resolution, capacity factor, tailing factor, theoretical plate, selectivity, retention volume and asymmetry factor of the peaks were calculated. Results illustrated in table (3-6)

Table (3-6): Analytical parameter for system suitability test of HPLC method.

Parameters	CPM	AMP	PPA	CAF
Theoretical plate (N)	1446.535	3166.313	2893.962	3784.711
Resolution (R_s)	5.800	1.000	3.449	-----
Tailing factor (TF)	1.000	0.938	0.905	0.920
Asymmetric factor(A_s)	1.000	1.091	1.333	1.106
capacity factor (K)	1.445	3.019	3.323	4.493
Selectivity (α)	2.089	1.100	1.353	-----
Retention time (t_R) in minute	3.423	5.627	6.052	7.69

The results indicate that the described method showed adequate column efficiency represented by (N) , good selectivity (α) , excellent correlation between peak area ration and concentration expressed in term of (A_s) and (T_f), acceptable (K') values, short retention time and good resolution between adjacent peaks for all analytes .The critical pair for this separation between AMP and PPA equal to 1.00 . The results obtained from system suitability tests were in agreement with the USP requirements [82] and could be applied for simultaneous determination of the four analytes.

(3-3) Influence of factors

(3-3-1) pH

The change of pH was tested by decreasing the buffer pH from (4.7) to strong acidic (2.8) which lead to protonation of the major drugs. The high polarity of the resulted compound decreased its (t_R) and sensitivity to the UV detector. Successfully applied a mobile phase with a pH 2.8 Adjustment was performed with the use of diluted 1M orthophosphoric acid. Even in such pH values, silanols are expected to be substantially ionized. The addition of an ion-pairing agent in the mobile phase results in a relative masking of their negative charge and therefore, in a decrease in the electrostatic attraction [83].

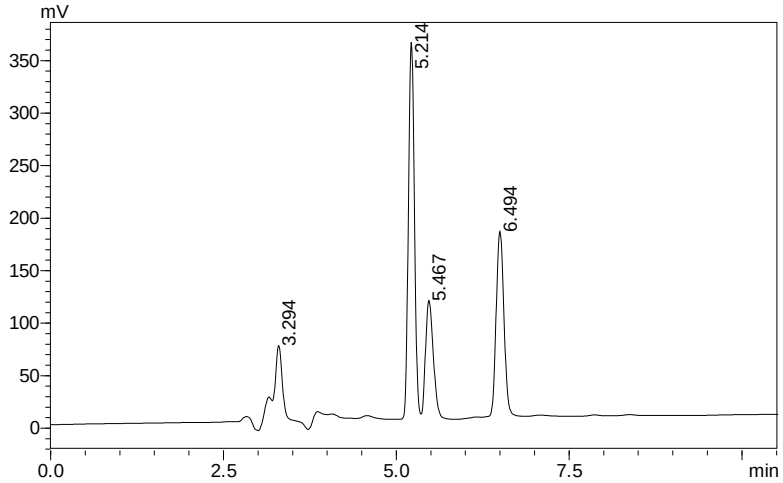


Figure (3-17): Chromatogram for standard solution of CP, PAR, PPA and CAF at pH 4.7

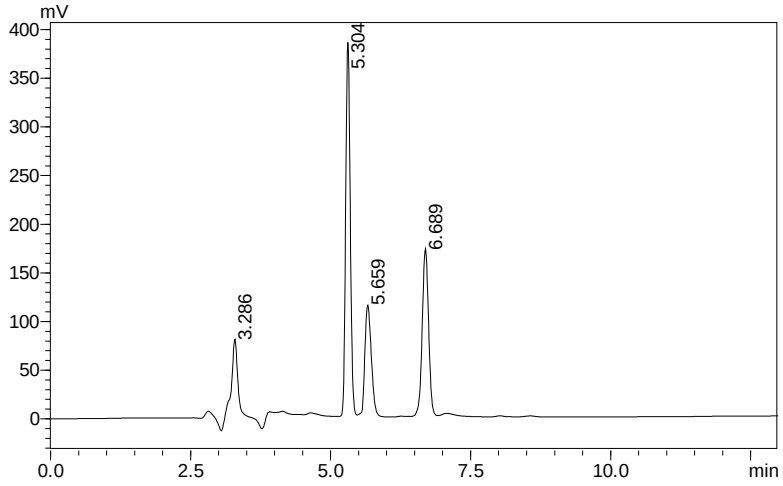


Figure (3-18): Chromatogram for standard solution of CP, PAR, PPA and CAF at pH 4.2

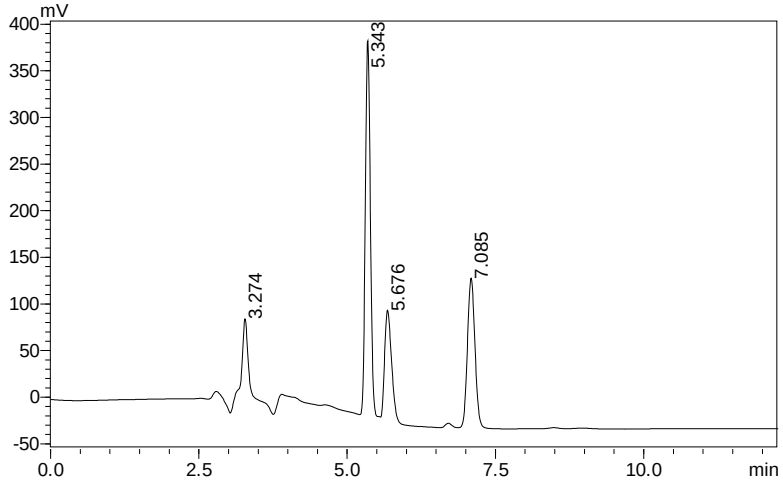


Figure (3-19): Chromatogram for standard solution of CP, PAR, PPA and CAF at pH 3.8

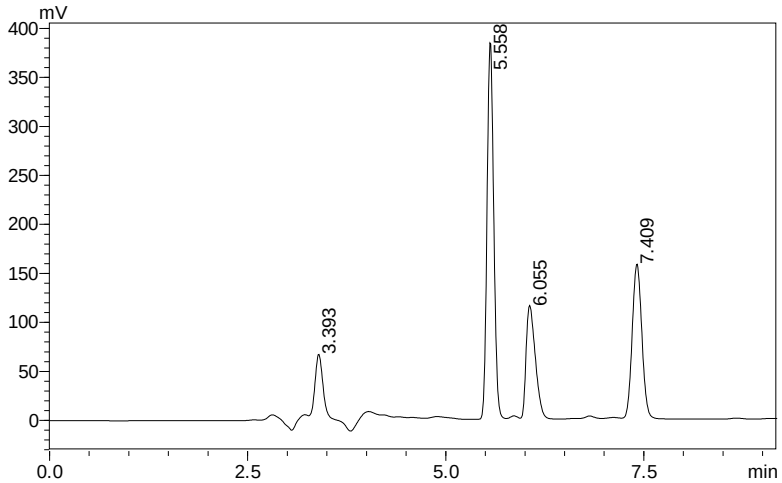


Figure (3-20): Chromatogram for standard solution of CP, PAR, PPA and CAF at pH 3.2

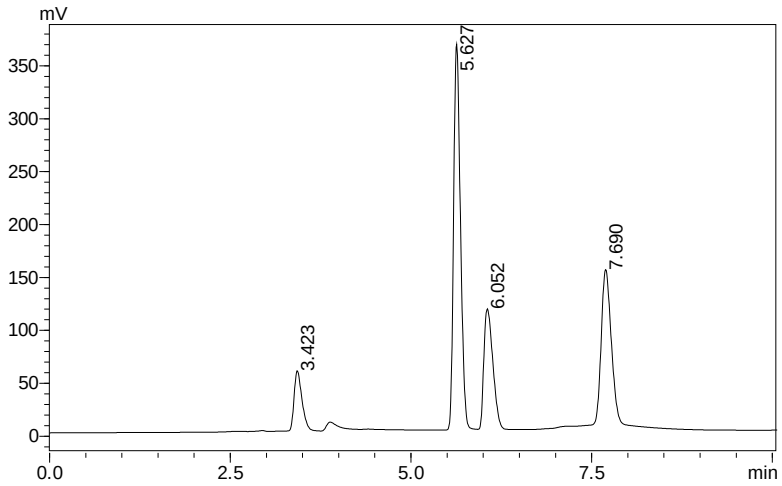


Figure (3-21): Chromatogram for standard solution of CP, PAR, PPA and CAF at pH 2.8.

Table (3-7): Effect of pH on separation of standard drugs.

pH	Area(n=3)		t_R (min)		R_S		K	
	CP	PAR	CP	PAR	CP	PAR	CP	PAR
4.2	346845.5	2173581.9	3.286	5.304	5.381	1.183	0.493	1.410
3.8	426526.7	2263825.9	3.274	5.343	5.517	1.110	0.488	1.428
3.2	420358.3	2253854.1	3.393	5.558	5.773	1.242	0.542	1.526
2.8	459253.9	2376737.6	3.423	5.627	5.800	1.200	0.556	1.558

Table (3-8): Effect of pH on separation of standard drugs.

pH	Area(n=3)		t_R (min)		R_S		K	
	PPA	CAF	PPA	CAF	PPA	CAF	PPA	CAF
4.2	845201.7	1336826.8	5.659	6.689	3.169	----	1.572	2.040
3.8	929225.8	1385213.6	5.676	7.085	3.522	-----	1.580	2.220
3.2	920922.8	1323409.4	6.055	7.409	2.850	----	1.752	2.367
2.8	967948.2	1403586.1	6.052	7.690	3.449	-----	1.755	2.496

The result in [figures (3-9), (3-10), (3-11), (3-12) and (3-13)] and tables [(3-7) and (3-8)] showed that pH had only a slight effect on retention time of four drugs. Resolution and Capacity factor improved when the pH reduced to 2.8; also the shape of CPM peak was improved in this pH. This may be due to ionized state of drugs at this pH [83].

(3-3-2) Temperature

As temperature increases from (25 – 40) ^{o}C, separation often worsens, while peak heights increase. Values of (k') usually decrease with increasing temperature according to the Van't Hoff equation [3], which can be expressed in HPLC as

$$Log\ k = A + \frac{B}{T_K} \qquad (3\text{-}1)$$

For a given solute and other conditions unchanged, A and B are temperature independent constants, and T_K is the temperature [3,69].

Operation at elevated temperatures decreases mobile phase viscosity and the column backpressure is decreased. Retention is observed to decrease with an increase in temperature.

Peak symmetry and good resolution were not achieved; hence temperature increased from (25 – 40) ^{o}C, also at high temperature the efficiency and column life is adversely affected. So 25 ^{o}C was selected for the present work. The results illustrated in figures [(3-14), (3-15), (3-16) and (3-17)] and table (3-9).

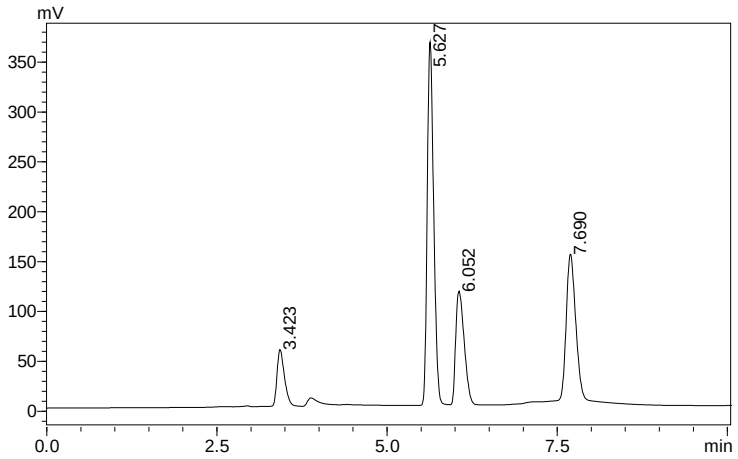


Figure (3-22): Chromatogram for standard solution of CP, PAR, PPA and CAF at 25 ^{o}C.

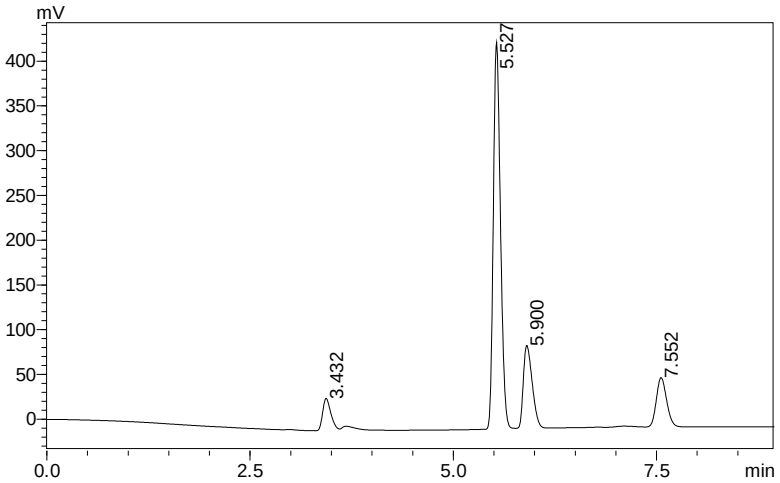


Figure (3-23): Chromatogram for standard solution of CP, PAR, PPA and CAF at 30^{o} C.

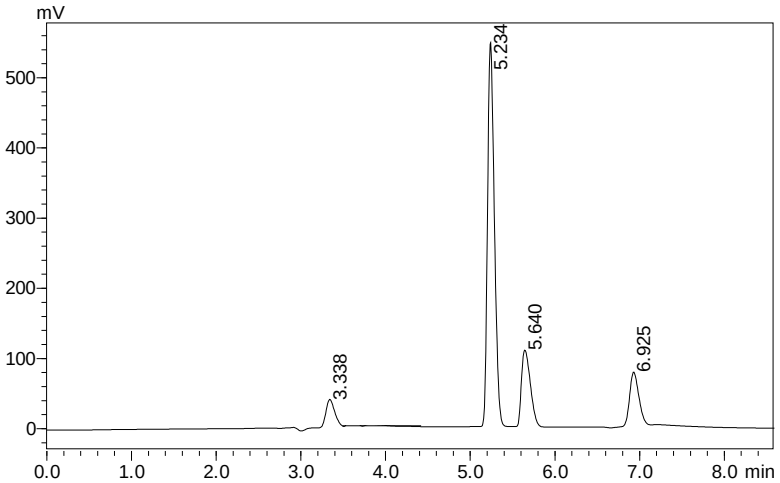


Figure (3-24): Chromatogram for standard solution of CP, PAR, PPA and CAF at 35 ^{o}C.

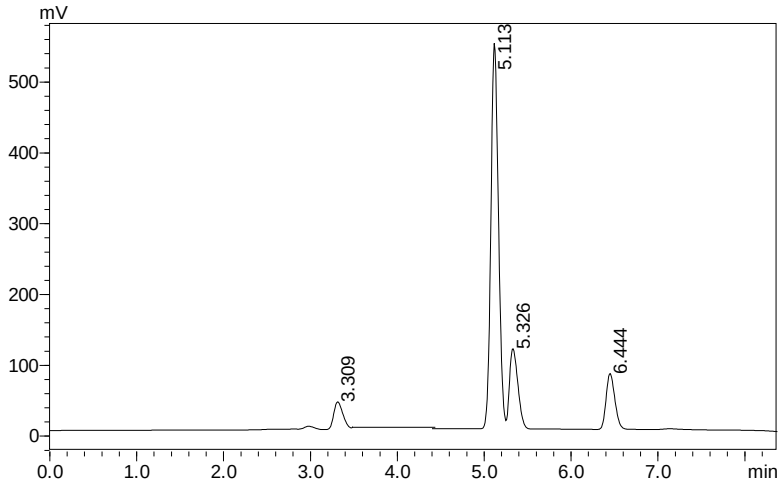


Figure (3-25): Chromatogram for standard solution of CP, PAR, PPA and CAF at 40 °C.

Table (3-9): Effect of temperature (°C) on Separation of CP, PAR, PPA and CAF.

Temperature (°C)	t_R (min)				K			
	CP	PAR	PPA	CAF	CP	PAR	PPA	CAF
25	3.423	5.627	6.052	7.690	0.556	1.558	1.755	2.496
30	3.423	5.527	5.900	7.552	0.530	1.513	1.682	2.433
35	3.599	5.234	5.540	6.925	0.517	1.379	1.564	2.160
40	3.309	5.113	5.236	6.444	0.501	1.324	1.421	1.929

(3-3-3) Flow rate

At 1 mL/min flow rate good resolution between CPM and PAR but long analysis time was observed. At 1.7 mL/min flow rate of overall analysis time was decreased with almost the same resolution between AMP and PPA and between PPA and CAF. The results were presented in figures [(3-18), (3-19), (3-20) and (3-21)] and table (3-10).

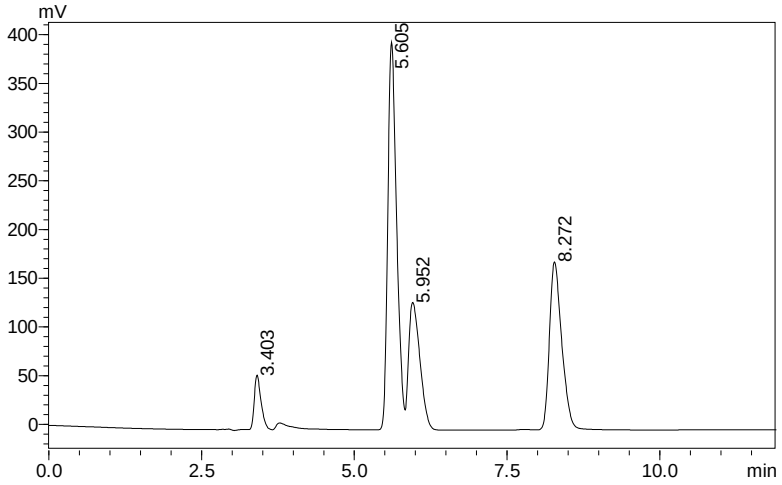


Figure (3-26): Chromatogram for standard solution of CPM, PAR, PPA and CAF at 1mL/min.

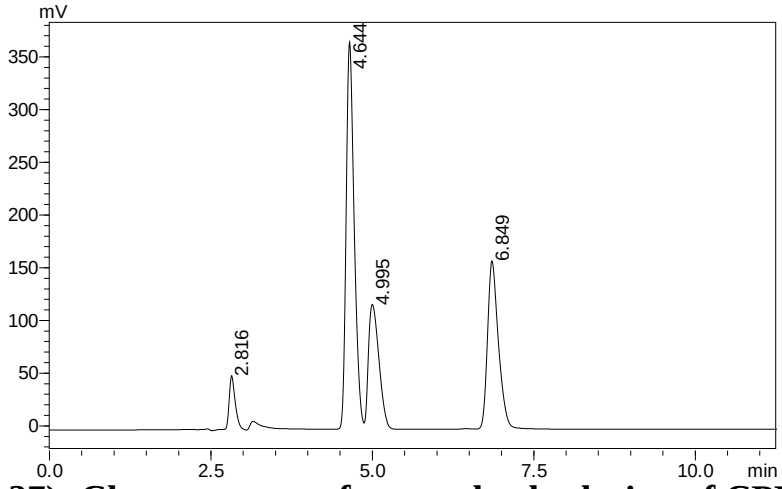


Figure (3-27): Chromatogram for standard solution of CPM, PAR, PPA and CAF at 1.2 mL/min.

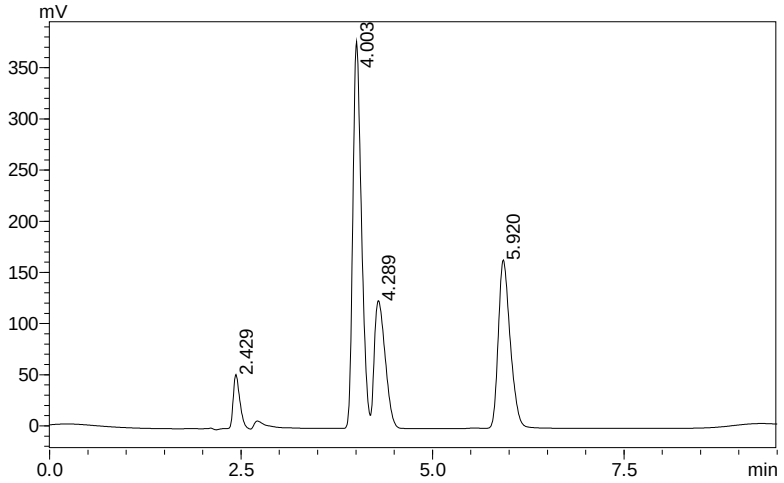


Figure (3-28): Chromatogram for standard solution of CPM, PAR, PPA and CAF at 1.4 mL/min.

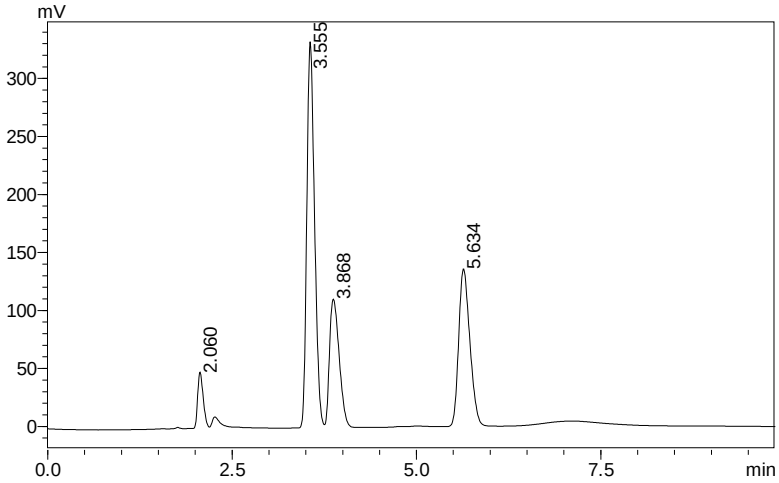


Figure (3-29): Chromatogram for standard solution of CP, AMP, PPA and CAF at 1.7 mL/min.

Table (3-10) Effect of Flow rate on separation of CPM, PAR, PPA and CAF.

Flow rate	**t_R (min)**				**R_S**			
mL/min	CPM	PAR	PPA	CAF	CPM	PAR	PPA	CAF
1	3.403	5.605	5.925	8.272	5.505	0.717	3.469	----
1.2	2.816	4.644	4.995	6.849	4.401	0.715	3.462	----
1.4	2.429	4.003	4.289	5.920	4.397	0.712	3.460	-----
1.7	2.034	3.557	3.868	5.634	4.271	0.709	3.532	-----

For the present study flow rate program from 1 mL/min (in 0-5 min) to 1.7 mL/min (after 5 min) was selected on the basis of less retention time, good peak shape, Acceptable back pressure , good resolution and better separation of drug [8].

(3-3-4) Volume injection

It was found that increase in the sample volume up to 20 µl led to an increase in the height of responses and baseline was achieved, so injection of 20 µl was selected to get high response without column overload and good baseline . The results illustrated in figures [(3-22), (3-23),(3-24) and (3-25)] and table (3-11).

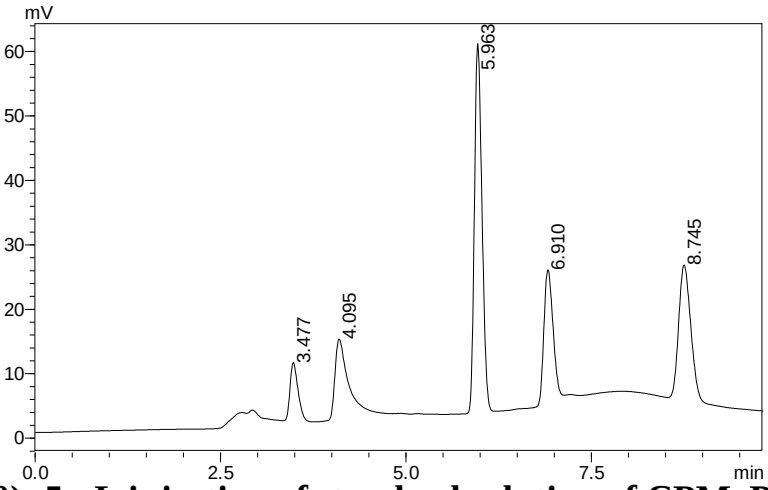


Figure (3-30): 5 µL injection of standard solution of CPM, PAR, PPA and CAF.

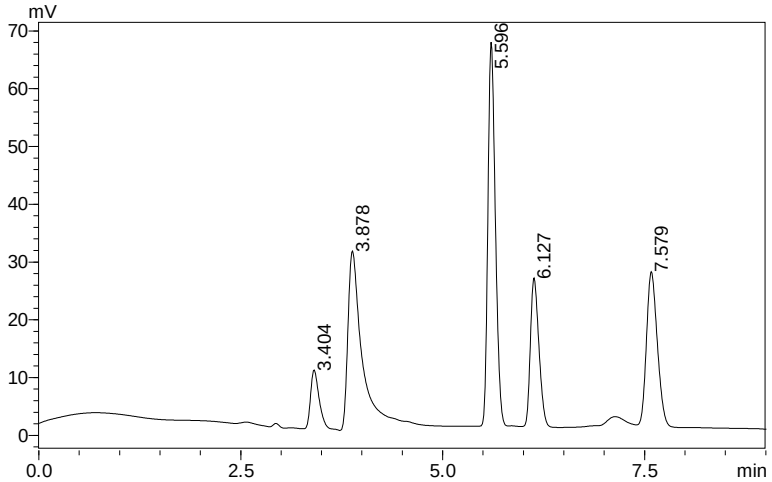


Figure (3-31): 10 µL injection of standard solution of CPM, PAR, PPA and CAF.

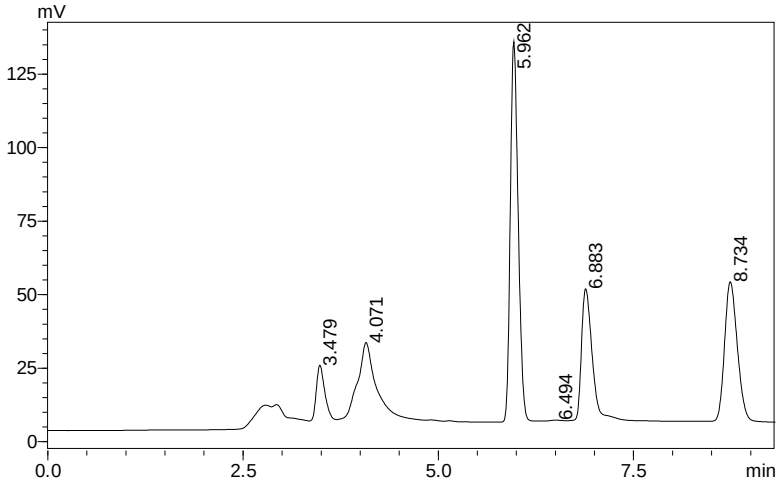


Figure (3-32): 15 µL injection of standard solution of CPM, PAR, PPA and CAF.

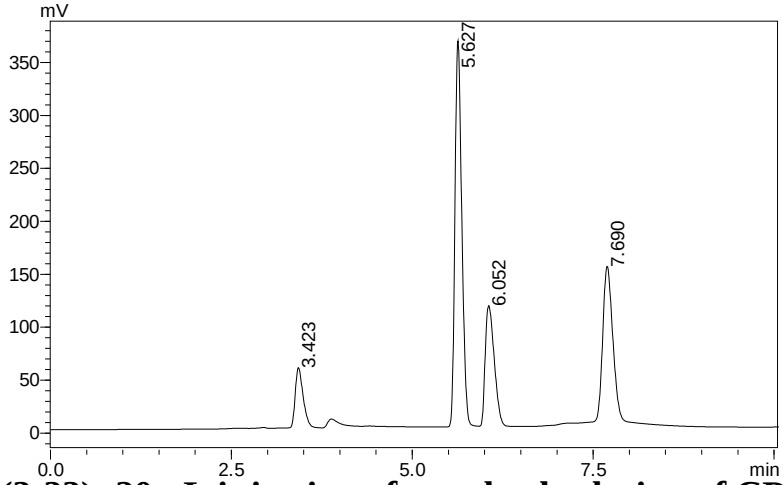


Figure (3-33): 20 µL injection of standard solution of CPM, PAR, PPA and CAF.

Table: (3-11) Effect of Volume injection.

Volume µL	Area (n=3)			
	CPM	PAR	PPA	CAF
5	67843.0	406973.8	170788.0	221261.5
10	72264.5	423952.2	189049.4	245727.6
15	132520.2	895431.5	388762.9	520797.7
20	429253.9	2376737.6	967948.2	1403586.1

For maximum sensitivity in trace analysis, sample as large as possible was injected, but too large a sample volume overloaded the column and result in distorted peaks with less increased in peak height. The sample volume was increased until resolution from neighboring overlapping component become limiting .

(3-4) Applications

Assay of pharmaceutical Dosage Form

The chromatographic method was applied to the determination of CPM, PAR, PPA and CAF in their pharmaceutical dosage form. Analysis was carried out using optimized mobile phase and HPLC conditions. Three batches of tablet were assayed. Because of the large difference between amount of CPM and PAR in the label claim for the tablet off-scale peak for AMP could result when the concentration of CPM was measurable. The results for CPM, PAR, PPA and CAF comparable with their corresponding labeled amounts and R.S.D % are shown in tables [(3-12) and (3-13)].

Table (3-12): Percentage recovery data for marketed drugs.

Recovery for drugs in tablet 1			
Drugs	**Labeled amount (µg/mL)**	**Amount found (µg/mL)**	**Recovery [%] (n=3)**
CPM	2.000	2.066	103.300
PAR	500.000	498.185	99.637
PPA	12.500	12.552	100.416
CAF	25.000	25.194	100.776

Recovery for drugs in tablet 2			
Drugs	**Labeled amount (µg/mL)**	**Amount found (µg/mL)**	**Recovery [%] (n=3)**
CPM	2.000	2.115	105.750
PAR	500.000	497.502	99.500
Recovery for drugs in tablet 3			
Drugs	**Labeled amount (µg/mL)**	**Amount found (µg/mL)**	**Recovery% (n=3)**
PAR	500.000	497.989	99.598
CAF	65.000	65.246	100.378

Table (3-13): Precision for marketed drugs.

Drugs in tablet 1	**(µg/mL)**	**R.S.D %(n=3)**
CPM	2.000	0.347
PAR	500.000	0.183
PPA	12.500	1.056
CAF	25.000	0.001
Drugs in tablet 2	**(µg/mL)**	**R.S.D %(n=3)**
CPM	2.000	0.052
PAR	500.000	0.259
Drugs in tablet 3	**(µg/mL)**	**R.S.D % (n=3)**
PAR	500.000	0.348
CAF	65.000	0.158

Results in tables [(3-12) and (3-13)] showed that the estimation of dosage form was accurate within the acceptable level.

(3-5) Recommendations

1- *Possibility to apply this method for the estimation of Chlorpheniramine maleate, Paracetamol, Phenylpropanolamine Hydrochloride and Caffeine in biological sample.*
2- *Possibility to apply this study to separate more than the four drugs under study.*

(3-6) Conclusion

1- *A reversed-phase HPLC method was developed and validated with UV detection for the simultaneous determination of CPM, PAR, PPA and CAF and proved to be more convenient and effective for the quality control of these drugs in pharmaceutical dosage forms.*
2- *The method gave good resolution for the drugs with a short analysis time below 8 minutes.*
3- *The method found to be simple, economical and useful with high accuracy, precision and low detection limit and quantitation limit. Rapidity and capability of quantifying very low concentration of respective drugs, made them useful for variety of analyses, including pure drug analysis and assay of formulations analysis.*
4- *The proposed methods did not utilize any extraction step for recovering the drug from the formulation excipients matrixes and their by decreased the degree of error, time in estimation of drugs and the overall cost of the analysis.*
5- *The solvent system used were simple mobile phase with isocratic elution and low buffer concentration compared to the reported method.*
6- *The good percentage recovery in tablet dosage forms suggests that the excipients present in the dosage forms have no interference in the determination. The percent R.S.D% was also less than 2% showing high degree of precision of the proposed method. So, the method is suitable for the determination of the drugs in tablets without interference from commonly used excipients, and could be used in a quality control laboratory for routine sample analysis.*

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Appendixes

$$S = \frac{\sqrt{(x_i - \bar{x})^2}}{N - 1}$$

Where ***S*** is the standard deviation, x_i is the experimental value, $\bar{x}$ is theoretical value, ***N*** number of values.

$$R.S.D\% = \frac{SD}{\bar{x}} \times 100$$

Where ***R.S.D %*** is the relative standard deviation, ***S*** is the standard deviation, $\bar{x}$ is theoretical value.

$$LOD = 3 \text{ x } \frac{SD}{S}$$

Where ***LOD*** is the Limit of detection, ***SD*** standard deviation of slop, ***S*** is the slope

$$LOQ = 10 \text{ x } \frac{SD}{S}$$

Where ***LOQ*** is the Limit of Quantification.

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

Where ***N*** is the number of theoretical plate, t_R is the retention time, ***W*** is the width of peak.

$$R = 2 \frac{t_{R,2} - t_{R,1}}{w_{2+} w_1}$$

Where ***R*** is the resolution factor, $t_{R,2}$ and $t_{R,1}$ are the retention time for adjacent peaks, W_2 and W_1 are the width of adjacent peaks.

$$TF = \frac{A + B}{2A}$$

Where ***TF*** is the peak tailing factor, ***B*** = peak width after the peak centre at 5 peak height and ***A*** = peak width at baseline before the peak centre.

$$A_S = \frac{A}{B}$$

Where A_s is the peak asymmetry factor, B = peak width after the peak centre at 10% peak height and A = peak width at baseline before the peak centre.

$$K = \frac{t_R - t_o}{t_o}$$

Where K is the capacity factor, t_R is the retention time, t_o is the void time.

$$\alpha = \frac{K_1}{K_2}$$

Where α is the selectivity, K_1 and K_2 are the capacity factors for adjacent peaks

$$E\% = \frac{|x_i - \bar{x}|}{x} \text{ x } 100$$

Where $E\%$ is the percentage error, x_i is the experimental value, $\bar{x}$ is the theoretical value.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية العلوم
قسم الكيمياء

التقدير والتقييم الآني لـ(كلورفينرامين مالييت ، باراسيتامول ، فنيل بروبانول امين هايدروكلورايد و الكافائين) في المستحضرات الصيدلانية الصلبة باستخدام كروموتوكرافيا العمود السائلة عالية الأداء- بالطور العكوس.

رساله مقدمة الى

كلية العلوم- جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل

احسان مهدي شهيد

بكالوريوس علوم كيمياء جامعة بابل-1994

بأشراف

الاستاذ المساعد الدكتور

عبد الباري مهدي ماهود

(1434 - 2013)

الخلاصة

كروموتوكرافيا الطور العكوس السائلة عالية الدقة طريقة تم تطويرها بنجاح لتقدير مزيج من اربعة أدوية هي كلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول أمين هايدروكلوريد و الكافائين بشكل آني. وقد وجد ان الطريقة بسيطة وحساسة وسريعة ودقيقة . تحقق الفصل باستخدام عمود فصل نوع Inertsil ODS-2 وبابعاد (250 ملم x 4.6 ملم ، وقطر جزيئة قدره 5 ملي مايكرو) وبدرجة حرارة 25 م^{o} . الطور المتحرك المستخدم هو مزيج من اسيتانيتريل – ماء – ميثانول – اورثوفوسفوريك اسيد (1 مولاري) تراي اثيل أمين – هكسان سلفونيك اسيد (14 ملي مول لتر$^{-1}$) كزوج ايوني –وبنسب حجمية (15 : 75 : 10 : 0.5 : 0.3: 4) على التوالي وتم تعديل الدالة الحامضية (pH) الى 2.8 باستخدام حامض السلفونيك المخفف. تم استخدام نظام جريان بسرعة ا مل / دقيقة (لمدة 5 دقيقة) الى 1.7 مل / دقيقة (بعد 5 دقيقة) لغاية 8 دقيقة وباستخدام كاشف الاشعة فوق البنفسجية وبطول موجي قدره 220 نانومتر . زمن الاحتجاز للكلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول امين هايدروكلوريد و الكافائين هو (3.423 ، 5.627 ، 6.052 و 7.690) دقيقة على التوالي . تم تقييم الطريقة عن طريق حساب الخطية ، الدقة ، الاستعادة ، التوافقية ، حد الكشف ، حد القياس الكمي والمتانة . الطريقة المقترحة كانت خطية ضمن حدود تراكيز (50 – 1) مايكروغرام / مل بمعامل ارتباط 0.9999 ،(500-1) مايكروغرام / مل بمعامل ارتباط 0.9999 ، (200 – 1) مايكروغرام / مل بمعامل ارتباط 0.9998 و (150- 1) ميكروغرام / مل بمعامل ارتباط 0.9998 للكلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول امين هايدروكلوريد و الكافائين على التوالي . ان تقييم الطريقة اعطى مستوات ممتازة للدقة والتوافقية مع مدى واطيء لمعيار الخطأ النسبي (1.5% >) و نسب استعادة ممتازة (101.650 – 101.640)% ، (100.350 – 99.484)% ، (100.751 – 100.840)% و (100.461 – 98.075)% للكلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول امين هايدروكلوريد و الكافائين على التوالي . حدود الكشف للادوية هي (0.516 ، 0.425 ، 0.466 و0.683) مايكرو غرام / مل اما حدود الكشف الكمية هي (1.720 ، 1.416 ، 1.552 و 2.277) مايكروغرام / مل للكلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول امين هايدروكلوريد و الكافائين على التوالي . كما تمت دراسة تاثير الدالة الحامضية ، درجة الحرارة ، حجم النموذج المحقون و سرعة الجريان على عملية الفصل . تم اختيار الدالة الحامضيه 2.8 ،

درجة الحرارة 20 م^{o} ، الحجم المحقون 20 مايكرولتر للعمل الحالي. نتائج هذه الدراسة اثبتت ان هذه الطريقة يمكن تطبيقها بنجاح لغرض تقدير اللكلورفينرامين ماليت ، باراسيتامول ، فنيل بروبانول امين هايدروكلوريد و الكافائين في المستحضرات الصيدلانية .

بسم الله الرحمن الرحيم

وَأَنْ لَيْسَ لِلْإِنْسَانِ إِلَّا مَا سَعَى (39)
وَأَنَّ سَعْيَهُ سَوْفَ يُرَى (40) ثُمَّ يُجْزَاهُ
الْجَزَاءَ الْأَوْفَى (41)

صدق الله العظيم