IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 10, Issue 4 Series. I (April 2020), PP. 17-45
www.iosrphr.org

Applying 3 Level Polynomial Design for Optimisation, Method Development and Validation for Ibuprofen and Caffeine By RP-UPLC Method

A Vikram[1], Dr R Shanmugam[1], Dr K Madhuri[1], Dr C.K Ashok Kumar[2]
[1]Department of Pharmaceutical Analysis, Sree Vidyanikethan College of Pharmacy,India.
[2]Department of Pharmacognosy, Sree Vidyanikethan College of Pharmacy.India.
Received 29 May 2020; Accepted 16 June 2020

Abstract:
Background: their exist several methods that uses combination of paracetamol and caffeine with the help of UPLC frame work. But with different combination of simple reverse phase Ultra performance liquid chromatography (RP-UPLC) method has been developed by statistical design of experiment for determination of Ibuprofen and Caffeine. These drugs are used as Non-steroidal anti-inflammatory drug (NSAID) and Central nervous system stimulant (CNS stimulant).
Materials and Methods: The separation was carried out using mobile phase consisting of Methanol and water (Adjusting with ortho phosphoric acid of P^H 4.5) ratio of 70:30 v/v. The column used is UHP ASB $C_{18,}$ (1.9µm, 2.1 X 50 mm) with flow rate of 0.1 ml/min using PDA detection at 254nm.The calibration curves were linear over a concentration range of 2001.2-6003.6 µg/mL and 325.2-975.6 µg/mL for Ibuprofen and Caffeine. The retention times of Ibuprofen and Caffeine were found to be 6.7 min and 1.8 min respectively.
Results: The regression coefficient was found to be 0.999 for Ibuprofen and Caffeine respectively. A design of experiment (methodology) was selected for the optimization and validation of the mobile phase composition. In addition, the method validation was done as per the ICH guideless using linearity, accuracy, precision, system suitability, and robustness as parameters.
Conclusion: The developed method gives an idea for research and development in method development that the factorial design can be applicable successfully for the method development and validation of Ibuprofen and Caffeine, which results in the decreasing the cost, time and manpower.The results of the study showed that the proposed RP-UPLC method is rapid, specific, precise and accurate and is useful for the routine analysis of Ibuprofen and Caffeine in bulk drug and in its pharmaceutical dosage form.
Keywords: Caffeine, Ibuprofen, RP-UPLC, Methanol, Ortho phosphoric acid, Design of experiment.

I. INTRODUCTION

ANALYTICAL CHEMISTRY:

Analytical chemistry is a branch of chemistry involved in separating, identifying and determining the relative amounts of the components making present in the matter.
It is mainly involved in the qualitative analysis or detection of compounds and quantitative analysis of the compounds. A qualitative method yields information about the identity of atomic or molecular species or functional groups in the sample. A quantitative method, in contrast provides numerical information as to the relative amount of one or more of these component [1].

CHROMATOGRAPHY:

Chromatography is relatively a new technique which was first invented by M.Tswett, a botanist in 1906. Chromatography was derived from Greek words chrome and graphos meaning “colour” and “writing” respectively. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyze to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation.

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analyzes in a mixture [2, 3].

Table no 1: Different type of Chromatographic technique

Basic principle involved	Type of Chromatography
Techniques by Chromatographic bed shape	Colunm Chromatography
	Paper Chromatography
	Thin layer Chromatography
Techniques by Physical state of mobile phase	Gas Chromatography
	Liquid Chromatography
Affinity Chromatography	Supercritical fluid Chromatography
Techniques by separation mechanism	Ion Exchange Chromatography
	Size Exclusion Chromatography
Special Techniques	Reversed Phase Chromatography
	Two-dimensional Chromatography
	Simulatedmoving-Bed Chromatography
	Paralysis Gas Chromatography
	Fast protein Liquid Chromatography

DIFFERENT BETWEEN HPLC, UPLC AND UFLC

The major different between HPLC, UPLC, and UFLC are given in table as below [4].

Table no 2: Differential between types of Liquid Chromatography

S. No.	Characteristics	HPLC	UPLC	UFLC
1.	Particle size	3 to 5µm	Less than 2 µm	2.2 µm
2.	Maximum back pressure	35-40 MPa	103.5 MPa	<35 MPa
3.	Analytical column	Alltima C_{18}	Acquity UPLC BEH C_{18}	Shim-pack XR columns
4.	Column dimensions	150 X 3.2 mm	150 X 2.1 mm	75 X 3.0 mm
5.	Column temperature	30°C	65°C	40°C
6.	Injection volume	5 µl (Std. In100% MeOH)	2µl (Std.In100% MeOH)	0.1-100 µl

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY:

UPLC refers to Ultra Performance Liquid Chromatography. Which improves chromatographic resolution, speed and sensitivity by the use of fine particle chemistry which saves time, reduces solvent consumption? Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis due to low diffusion coefficients in liquid phase, involving slow diffusion of analytes in the stationary phase. UPLC is a derivative of HPLC whose underlying principle is that as column packing particle size decreases, efficiency and thus resolution increases. As the particle size decreases less than 2 µm, the efficiency shows a significant gain and it does not diminish at increased linear velocities or flow rates according to the common Van Deemter equation By making use of the smaller particles, the speed of analysis and peak capacity i.e., number of peaks resolved per unit time, can be prolonged to the maximum values and these values are much better than the values achieved earlier by HPLC. Over many years, researchers have looked at "fast LC" with accuracy as a way to speed up analysis [10].

Principle:

The UPLC is based on the principle of use of stationary phase consisting of particles less than 2um. The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). The Van Deemter curve, governed by an equation with three components shows that the usable flow range for a good efficiency with small diameter particles is much greater than for larger diameters.

$H = A + B/v + Cv$
Where:
A, *B* and *C* are constants
V is the line ar velocity, the carrier gas flow rate.
A term is independent of velocity and represents “eddy” mixing. It is smallest when the packed column particles are small and uniform.

B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by *v*.

C term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again [5-9].

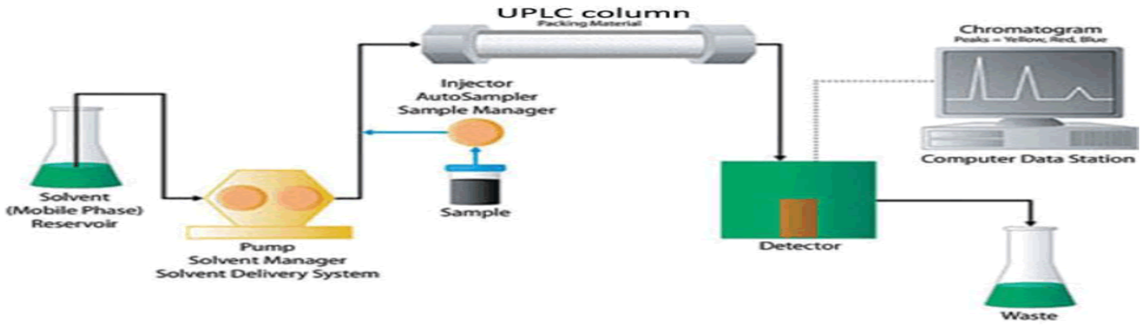


FIG. 1. **Working of UPLC.**

Figure no 1: Working of UPLC

ANALYTICAL METHOD DEVELOPMENT

The number of drugs introduced into the market is increasing every year. These Drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens the possible uncertainty’s in the continuous and wider usage of these drugs, reports of new toxicities (Resulting in their withdrawal from the market).Development of Patient resistance and introduction of better drugs by competitors, under these conditions, standards and analytical procedures for these drugs may not be available in the Pharmacopeia, it becomes necessary, therefore to develop newer analytical methods for such drugs [11, 12].

METHOD VALIDATION

Analytical Method Validation can be defined as (ICH) “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”.
Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

EXPERIMENTAL DEIGNS STUDIES

Experimental designs

Experimental designs are being often used for the optimization of several operating conditions of various processes and for improving the chromatographic separation performance, as well as attaining high extraction efficiency. Ideally, a number of factors have simultaneous effect on a process. Nevertheless, identification and optimization of significant factors as a function of experimental design is most effective to achieve a competent result by fewer experimental trials. Consequently, the experimental design can be well defined as an approach to solve the problems systematically and obtain information-rich result [13].Optimum and valid results with a minimum effort, time and resources are the primary objectives of applying the experimental design in analytical process [19].In an experimental design, one or several predetermined factors are deliberately maneuvered to perceive their influence on the experimental outcome. Based on the objectives of an experiment, all the designs can be classified into two broad categories: Screening Designs, Response Surface Designs (optimization design).

Screening designs

Since a huge number of factors influence the UPLC process, some of them that do not have significant effect on it must be discarded. Screening of the most influential factors becomes the primary objective of employing experimental design in UPLC. These designs are used with a purport to identify the most important factors and their interactions from all potential factors. They are very useful to examine qualitative, quantitative and mixer-related factors simultaneously[20].From the literature it is evidenced that Full Factorial designs (FFD), Fractional Factorial designs (FrFD) and Plackett-Burman designs (PBD) are frequently used as screening designs [21-23].such two-level designs allow screening of high number of factors with fewer experiments. Analysis of variance (ANOVA) or regression analysis can be the basis for computing effect of the studied factors on a particular response. They are frequently applied for improvement of separation techniques, formulations, products or processes of quality control and robustnessand ruggedness [14, 15].the steps to be performed in such designs are identical to that of robustness or ruggedness test with the discrepancy in the intervals within the two levels of the factors. Several applications of three or more or mixed-level screening designs also has been evidenced from the literature.

Response surface designs

Optimization is an additional practice of chemometric approach that endorses the optimal condition or settings of a process. Such approach usually proceeds with a screening design to select the potential factors.Response surface designs are of two types: symmetrical designs and asymmetrical designs. Three-level FFD, Central Composite design (CCD) and Box-Behnken design (BBD), Taguchi design (TD), and Doehlert designs cover a symmetrical domain with a center point to estimate experimental error. Asymmetrical designs such as D-optimal design form an asymmetrical shape when an asymmetrical experimental domain is examined. Such designs can also form a symmetrical shape in a symmetrical domain. Mixture designs are applied to study mixture variables only, i.e. to optimize the composition of mixture. ANOVA, signal-to-noise ratio and range analysis are the basis of the statistical analysis methods for response surface designs. Range analysis is used to find the effect of each factor and determine the optimal level of different factors. For a factor, the range of means is the difference of the maximum and minimum means of all levels. For a system, the factor with the largest range of means has the strongest influence on the performance. Range analysis can find the optimal value of different factors but this method cannot clearly and quantitatively determine the significance of different factors. In the ANOVA, the data are analyzed by a F-test. The F value of each factor implies the ratio of the variance for the each factor to that of the experimental error. The percentage contribution of each factor is the percentage of the sum of square deviation due to that factor in the total sum of square deviation. It reflects the factor's influence. Regression analysis enables to estimate the relationships among variables via a regression function. Linear first order and second order models are quite common. A fruitful implementation of experimental design in UPLC can be executed through four common stages; i.e.: (i) choosing the convenient design, (ii) suitable software, (iii) experimental trials, data analysis, and (iv) interpretation[16, 17].

Application of Design of Experiment

DOE (Design of Experiments) provides a powerful means to achieve breakthrough improvements in product quality and process efficiency. From the viewpoint of manufacturing fields, this can reduce the number of required experiments when taking into account the numerous factors affecting experimental results. DOE can show how to carry out the fewest number of experiments while maintaining the most important information. The most important process of the DOE is determining the independent variable values at which a limited number of experiments will be conducted. For this purpose, Taguchi proposed an improved DOE. This approach adopts the fundamental idea of DOE, but simplifies and standardizes the factorial and fractional factorial designs so that they conducted experiments can produce more consistent results. The major contribution of the work has been in developing and using a special set of orthogonal arrays for designing experiments. Orthogonal arrays are a set of tables of numbers, each of which can be used to lay out experiments for a number of experimental situations. The DOE technique based on this approach makes use of these arrays to design experiments. Through the orthogonal arrays, it is possible to carry out fewer fractional factorial experiments than full factorial experiments. Also, the relative influence of factors and interactions on the variation of results can be identified. Through fractional experiments, optimal conditions can be determined by analyzing the S/N ratio (Signal-to-Noise ratio) as a performance measure, often referred to as ANOVA (Analysis of Variance). The details of this approach are presented in the following subsections [18].

Method development and validation for Ibuprofen and caffeine by RP-UPLC method was performed because before developed method was with combination drug with Ibuprofen and Famotidine and by Drotaverine Hydrochloride and Ibuprofen using different columns, mobile phase ratios, wavelength.

II. REVIEW OF LITERATURE

- **Bikash Ranjan jena et al.,(2017)** Had investigated and developed UPLC method and validated for the simultaneous estimation of paracetamol and caffeine capsule dosage form using c18 column 1.7µm with 0.1%w/v H_3PO4 & 100%v/v buffer as mobile phase A and methanol & acetonitrile(50:50) as mobile phase B. The maintained Chromatographic conditions are flow rate of 0.5ml/min, PDA detector, column temperature 40^0c, detection wavelength 275nm, and injection volume 2µL. The retention time paracetamol and caffeine was found to be 0.6 and 1.7 respectively.

- **Mittal A et al., (2016)** Developed A simple and precise RP-HPLC method was developed and validated for the simultaneous determination of amlodipine and valsartan combination in bulk and tablet dosage form. This method involves the design of experiments approach for the optimization of mobile phase by taking methanol, pH and flow rate as the dependent variable and their effect was seen on retention time of amlodipine (4.35min) and valsartan (10.26 min). A linear response was observed over the concentration range of 5–50 µg/mL for amlodipine and 10-100µg/ mL for valsartan.. The method was successfully validated in accordance with ICH guideline acceptance criteria for linearity, accuracy, precision, specificity, robustness. The analysis concluded that the method was selected for simultaneous estimation of amlodipine and valsartan, further can be potentially used for estimation of these drugs in combined dosage form.

- **Rekulapally VijayKumar et al.,(2015)** Have developed a novel, rapid validated stability indicating RP-UPLC method for the estimation of Drotaverine Hydrochloride and ibuprofen impurities in oral solid dosage form using Waters UPLC BEH C18, 100 × 2.1 mm, 1.7 µm column. Mobile phase A and B comprises phosphate buffer and acetonitrile of 900:100v/v & 400:600v/v ratio respectively. The chromatographic conditions maintained are flow rate 0.3ml/min; detector wavelength 210nm, injection volume is 1.0 µL. The impurities in both the samples found to be below 5 %(should be less than 10%).

- **Rafael R.Cunha et al., (2015)** Reported the propose two new methods for simultaneous determination of paracetamol, caffeine and Ibuprofen in pharmaceutical formulations. One method is based on high-performance liquid chromatography with diode-array detection and the other on capillary electrophoresis with capacitively coupled contactless conductivity detection. The separation by high-performance liquid chromatography with diode-array detection was achieved on a C_{18} column (250×4.6 mm^2, 5 µm) with a gradient mobile phase comprising 20–100% acetonitrile in 40 mmol L^{-1} phosphate buffer pH 7.0. The separation by capillary electrophoresis with capacitively coupled contactless conductivity detection was achieved on a fused-silica capillary (40 cm length, 50 µm i.d.) using 10 mmol L^{-1} 3, 4-dimethoxycinnamate and 10 mmol L^{-1} β-alanine with pH adjustment to 10.4 with lithium hydroxide as background electrolyte. The determination of all three pharmaceuticals was carried out in 9.6 min by liquid chromatography and in 2.2 min by capillary electrophoresis. Detection limits for caffeine, paracetamol and ibuprofen were 4.4, 0.7, and 3.4 µmol L^{-1} by liquid chromatography and 39, 32, and 49 µmol L^{-1} by capillary electrophoresis, respectively. Recovery values for spiked samples were between 92–107% for both proposed methods.

- **Venkata Raveendra Babu Vemula et al.,(2013)**Reportedthe HPLC determination was carried out on an Agilent XDB C-18 column (4.6 x 150mm, 5 µ particle size) with a gradient mobile phase composed of 0.1 % orthophosphoric acid and acetonitrile at a ratio of: 0.01/95/5, 2.5/95/5, 6/10/90, 8/10/90, 8.1/95/5 and 13/95/5 for time (min)/0.1 % orthophosphoric acid (%)/acetonitrile (%) at a flow rate of 1.0 mL/min. Column temperature was maintained at 30 °C and detection was carried out using a photodiode array (PDA) detector at 210 nm. Validation parameters including system suitability, linearity, precision, accuracy, specificity, limit of detection (LOD), and limit of quantification (LOQ), stability of sample and standard stock solutions as well as robustness were obtained as per International Conference on Harmonization (ICH) guidelines. The proposed method was applied to the determination of phenylephrine and Ibuprofen in commercial tablets.

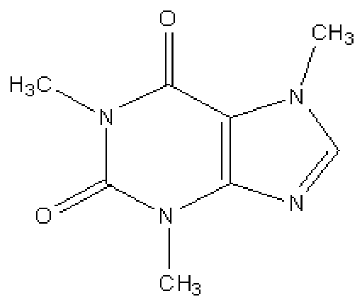
- **Christian et al., (2013)** developed a high performance liquid chromatographic method was developed and validated for the quantitative determination of brinzolamide and brimonidinetartrate. Employing an isocratic RP-HPLC Phenomenex C18 (5 µm, 250×4.6 mm) column resulted in an adequate separation for brinzolamideand brimonidine tartrate with retention time of 5.7±0.345 and 3.8±0.568 min, respectively. Best resolution was achieved with the phosphate buffer (pH 6.6): acetonitrile: methanol (45:15:40) as mobile phase pumped at the flow rate of 1.0 ml/min with the detection wavelength of 254 nm. Regression coefficient for both brinzolamide and brimonidine tartrate was found to be 0.9993 and 0.9965, respectively indicating linearity within the concentration range. Fractional factorial design, 24-1 was applied to assess the robustness of the developed method. Various independent variables selected for robustness testing were wavelength, acetonitrile volume in the mobile phase, flow rate and pH of the mobile phase. It was statistically evinced that retention time of drugs without the loss of resolution between two drugs, is affected by varying the independent variables flow rate and acetonitrile volume in the mobile phase from minimum to maximum. Hence, the limits must be strictly defined for the method conditions; flow rate and acetonitrile volume in mobile phase for optimum separation of drugs with acceptable retention time and resolution. The validation parameters like linearity, precision, accuracy, limit of detection and limit of quantitation were also found to be suitable. The proposed method can hence be successfully applied to quantify brinzolamide and brimonidine tartrate during quality control of formulation.

- **Boyka tsvetkova et al., (2012)** Have Development and validation of a high-performance liquid chromatographic analytical procedure for simultaneously determination of paracetamol and caffeine in a tablet formulation. The separation was achieved on a C18 column at a flow rate of 1.5 ml/min with UV detection at 220 nm. The mobile phase was composed of 1mM phosphate buffer pH 3.0 – acetonitrile (85:15 *v/v*) containing 0.2 % triethylamine (*v/v*). The method was validated for analytical parameters specificity, linearity, precision, accuracy, LOD, LOQ and robustness. The linearity of the method was investigated in the concentration ranges 31.25-250 μg/ml (r = 0.9999) for paracetamol and 4.06-32.50 μg/ml (r = 0.9986) for caffeine. Mean recoveries for paracetamol and caffeine were 99.37 and 99.12 %, respectively. The analytical procedure was applied to identification, purity and assay tests on model drug formulation. It was established that the developed analytical procedure was successfully used for routine analysis of paracetamol and caffeine in model drug dosage form without any interference from included excipients.

- **Reddy YR et al., (1999)** reported a RP-UPLC method and validated for simultaneous estimation of Ibuprofen and Famotidine in pharmaceutical dosage form. In this method separation was achieved on Acquity UPLC BEH C-18, 50 mm × 2.1 mm and 1.7 μm column with gradient elution and mobile phase (A) containing a mixture of sodium acetate buffer and methanol in ratio of 85:15v/v, mobile phase (B) containing a mixture of sodium acetate buffer and methanol in the ratio of 25:75v/v, flow rate of 0.3ml/min, column temperature -25^{0}C, injection volume 1.5μL at the wavelength 260nm. The developed method was found to be more stable.

III. DRUG PROFILE

Drug Name: Caffeine
Chemical Structure:



IUPAC Name: 1, 3, 7-Trimethylpurine-2, 6-Dione
Molecular Formula: $C_8H_{10}N_4O_2$
Molecular Weight: 194.19 g/mol
Solubility: Methanol
Category: CNS Stimulant
PHARMACOLOGY:
Pharmacodynamics

In the absence of caffeine and when a person is awake and alert, little adenosine is present in (CNS) neurons. With a continued wakeful state, over time it accumulates in the neuronal synapse, in turn binding to and activating adenosinereceptors found on certain CNS neurons; when activated, these receptors produce a cellular response that ultimately increases drowsiness. When caffeine is consumed, it Santagonizes adenosine receptors; in other words, caffeine prevents adenosine from activating the receptor by blocking the location on the receptor where adenosine binds to it. As a result, caffeine temporarily prevents or relieves drowsiness, and thus maintains or restores alertness.

Pharmacokinetics

Caffeine from coffee or other beverages is absorbed by the small intestine within 45 minutes of ingestion and distributed throughout all bodily tissues. Peak blood concentration is reached within 1–2 hours it is eliminated by first-orderkinetics. Caffeine can also be absorbed rectally, caffeine (for the relief of migraine) and chlorobutanol and caffeine (for the treatment of hyperemesis). However, rectal absorption is less efficient than oral: the maximum concentration and total amount absorbed are both about 30% of the oral amounts.

Absorption

Readily absorbed after oral or parenteral administration. The peak plasma level for caffeine range from 6-10mg/L and the mean time to reach peak concentration ranged from 30 minutes to 2 hours.

Protein binding

Low (25 to 36%)

Metabolism

Hepatic cytochrome P450 1A2 (CYP 1A2) is involved in caffeine biotransformation. About 80% of a dose of caffeine is metabolized to paraxanthine (1, 7 –dimethylxanthine), 10% to theobromine (3, 7 –dimethylxanthine), and 4% to theophylline (1, 3-dimethylxanthine).

Mechanism of action

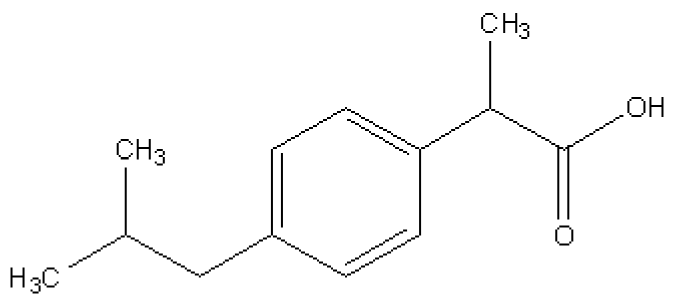
Caffeine is a methylxanthine alkaloid found in the seeds, nuts, or leaves of a number of plants native to South America and East Asia that is structurally related to adenosine and acts primarily as an adenosine receptor antagonist with psychotropic and anti-inflammatory activities. Upon ingestion, caffeine binds to adenosine receptors in the central nervous system (CNS), which inhibits adenosine binding. This inhibits the adenosine-mediated downregulation of CNS activity; thus, stimulating the activity of the medullary, vagal, vasomotor, and respiratory centers in the brain. This agent also promotes neurotrasmitter release that further stimulates the CNS. The anti-inflammatory effects of caffeine are due to nonselective competitive inhibition of phosphodiesterases (PDE_S). Inhibition of phosphodiesterases raises the intracellular concentration of cyclic AMP ($_C$AMP), activates protein kinase A, and inhibits leukotriene synthesis, which leads to reduced inflammation and innate immunity.

Side effects

Adverse effects includeAnxiety, Insomnia, Vomiting, Nausea, Rapid heartbeat, Digestive issue.

Drug Name: Ibuprofen

Chemical Structure:



IUPAC Name: (RS)-2-(4-(2-Methylpropyl) phenyl) propionic acid

Molecular Formula: $C_{13}H_{18}O_2$

Molecular Weight: 206.29 g/mol

Solubility: Methanol

Category: Nonsteroidal anti-inflammatory drug (NSAID)

PHARMACOLOGY:

Pharmacodynamics

Ibuprofen is a nonsteroidal anti-inflammatory agent (NSAIA) or nonsteroidal anti-inflammatory drug (NSAID), with analgesic and antipyretic properties. Ibuprofen has pharmacologic actions similar to those of other prototypical NSAIAs, which are thought to act through inhibition of prostaglandin synthesis.

Pharmacokinetics
Contrary to pharmacodynamics, is what your body does with the medication once it enters the body, in other words, where does it go? When taken by mouth ibuprofen begins to work within one-half to one hour and reaches its maximum effectiveness in about two to four hours. Each dosing may continue to exert its effects from six to eight hours.

Absorption
~ 80% absorbed from GI tract
Time to reach peak plasma concentration = 47 minutes (suspension), 62 minutes (chewable tablets), 120 minutes (conventional tablets)

Protein binding
90-99% to whole human plasma and site II of purified albumin, binding appears to be saturable and becomes non-linear at concentrations exceeding 20 mcg/ml.

Metabolism
R-enanatiomer undergoes extensive enantiomeric conversion (53-65%) to the more active S-enantiomer *in vivo*. Metablized by oxidation to 2 inactive metabolites: (+)-2[4´-(2-hydroxy-2-methylpropyl) phenyl] propionic acid and (+)-2-[4´-(2-carboxypropyl) phenyl] propionic acid. Very small amounts of 1-hydroxyibuprofen and 3-hydroxyibuprofen have been recovered from urine. Cytochrome P450 2C9 is the major catalyst in the formation of oxidative metabolites. Oxidative metabolites may be conjugated to glucuronide prior to excretion.

Mechanism of action:
Ibuprofen is a non-selective inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis via the arachidomic acid pathway. Its pharmacological effects are believed to be due to inhibition cyclooxygenase-2 (COX-2) which decreases the synthesis of prostaglandins involved in mediating inflammation, pain, fever and swelling. Antipyretic effects may be due to action on the hypothalamus, resulting in an increased peripheral blood flow, vasodilation, and subsequent heat dissipation. Inhibition of COX-1 is thought to cause some of the side effects of Ibuprofen including GI ulceration. Ibuprofen is administered as a racemic mixture. The R-enantiomer undergoes extensive interconversion to the S-enantiomer in vivo. The S-enantiomer is believed to be the more pharmacologically active enantiomer.

Side effects
Adverse effects include nausea, dyspepsia, diarrhea, constipation, gastrointestinal ulceration/bleeding, headache, dizziness, rash, high blood pressure.

IV. EXPERIMENTAL METHODOLOGY

INSTRUMENTS USED

Table no 3: List of instruments used

SL.No	Instrument	Model	Make
1	UPLC	LC2030	Shimadzu, Japan
2	UV/VIS Spectrophotometer	UV-1700	Shimadzu, Japan
3	Weighing Balance	AUW220D	Shimadzu, Japan
4	Sonicator	NA	PCA Analytic

CHEMICALS USED:

Table no 4: List of chemicals used

SL.No	Chemical	Brand	Grade
1	Methanol	Finar, Ahmedabad	HPLC
2	Ortho-Phosphoric Acid	Rankem, Mumbai	HPLC
3	Con.HydrochloricAcid	Rankem, Mumbai	AR
4	Hydrogen Peroxide	Fisher, Ahmedabad	AR
5	Sodium Hydroxide	Rankem, Mumbai	AR

WORKING STANDARD / REFERENCE STANDARD

Table no 5: List of working standard used

SL.No	Reference Standard / Working Standard	Brand	Grade
1	Caffeine	Strides shasun, Pondicherry	NA
2	Ibuprofen	Strides shasun, Pondicherry	NA

Factorial Design Study

Design expert 11 software was initialized with number of designs among them three level polynomial design is selected. Where three factors and three responses are associated with the 27 runs and the values of factors and responses obtained from literature review were entered in the design. Therefore each response, For example Retention time (Rt) was measured and recorded by analyzing the P-value were found to be significant and perturbation plots, perturbation vs actual and 3D surfaces regarding the interaction of the R_1R_2, R_1R_3, and R_1F_1, R_1F_2, R_1F_3 were obtained. From this the

Design is to be confirmed by point prediction through 100 solutions, in which the desirability containing '1' was most probably preferred.

METHODOLOGY

Trial-1:

The trial was performed using mobile phase of methanol and water (P^H 4.5, adjusted with orthophosphoric acid) in the ratio of 50:50 v/v with a flow rate of 0.2 ml/ min at different detection wavelengths 252nm, 254 nm, and 256 nm respectively. The analysis was performed on a PDA detector, with a stationary phase UHP ASB C_{18}, 2.1×50mm, 1.9 µm on UPLC system.

Trial-2:

The trial was performed using mobile phase of methanol and water (P^H 4.5, adjusted with orthophosphoricacid) in the ratio of 60:40 v/v with a flow rate of 0.3 ml/ min at different detection wavelengths 252nm, 254 nm, and 256 nm respectively. The analysis was performed on a PDA detector, with a stationary phase UHP ASB C_{18}, 2.1×50mm, 1.9 µm on UPLC system.

Trial-3:

The trial was performed using mobile phase of methanol and water (P^H 4.5, adjusted with orthophosphoricacid) in the ratio of 70:30 v/v with a flow rate of 0.1 ml/ min at different detection wavelengths 252nm, 254 nm, and 256 nm respectively. The analysis was performed on a PDA detector, with a stationary phase UHP ASB C_{18}, 2.1×50mm, 1.9 µm on UPLC system.

OPTIMISED CHROMATOGRAPHIC CONDITION

The method development for Ibuprofen and Caffeine was optimized with chromatographic condition by mobile phase ratio methanol and water (P^H 4.5, adjusted with orthophosphoricacid) in the ratio of 70:30 v/v with a flow rate of 0.1 ml/ min , detection wavelength at 254nm, run time was obseverd in 15min.

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Mobile Phase:

About 300 ml of water and 700 ml of methanol was taken, adjusted to P^H 4.5 with orthophosphic acid and kept in the ultrasonication for 10 min and was filtered through 0.45µ.

Diluent Preparation:

About 700ml of methanol and 300ml of water (P^H 4.5, adjusted with orthophosphoric acid) was mixed and sonicated for 5 min.

PREPARATION OF STANDARD AND SAMPLE SOLUTION:

Standard Preparation:

About 32.5mg of Caffeine and 200mg of Ibuprofen was taken in a 50ml volumetric flask, to that 20ml of diluent was added and sonicated for 15 min to dissolve. Cooled to room temperature made up volume with diluent.

Sample Preparation:

About 1020 mg of sample was taken in a 100ml volumetric flask, to that added 50ml of diluent and sonicated for 15 min to dissolve. Cooled to room temperature made up the volume with diluents, then filtered the solution through 0.45μm nylon filter.

METHOD VALIDATION PARAMETER

- System suitability
- Specificity
- Linearity and range
- Accuracy
- Precision
  - Method precision
  - Intermediate precision
- Robustness
- Ruggedness
- Filter validation
- Solution stability

SYSTEM SUITABILITY:

Standard solution was prepared by using Standard stocks and five replicate injections are given into the UPLC system. The system suitability parameters were calculated from standard chromatograms by evaluating the % RSD from five replicate injections of Standard stock**,** retention times and peak areas.

Acceptance criteria:

The tailing factor of Caffeine and Ibuprofen should be NMT 2.0

The theoretical plate of Caffeine and Ibuprofen should be NLT 2000.

SPECIFICITY:

Solutions of blank, standard and sample were prepared and are injected into chromatographic system.in order to improve the specificity and selectivity of the method the sample and standard peaks Rt (min) was recorded.

Acceptance criteria:

There should not be any interference by blank, placebo with the main analyte peak at specified wavelength.

LINEARITY:

A Serial solutions of Ibuprofen and Caffeine was prepared by using Standard stock solutions to get concentration levels from 50%, 75%, 100%, 125% and 150% by pipetting 5ml, 7.5ml, 10ml, 12.5ml, and 150ml stock solution was taken into 100 ml volumetric flasks respectively and made up to the volume with diluent. Measured the peak area response of solutions. The calibration curve was plotted between concentration and peak area. Correlation coefficient %RSD was calculated.

Acceptance criteria:

Correlation coefficientshould be not less than 0.999.

% of y- Intercept should be ±2.0.

ACCURACY:

Accuracy was performed at 3 levels that are 50% level, 100% level, 150% level in triplicate at each level.

Accuracy 50% solution:

200 mg of Ibuprofen and 32.5 mg Caffeine sample was weighed and transferred into 100 ml volumetric flask, add 50ml of diluent and sonicate for 15 min to dissolve and makeup to the volume (100ml) with dilute and filter through 0.45µ filter.

Accuracy 100% solution:

400 mg of Ibuprofen and 65 mg Caffeine sample was weighed and transferred into 100 ml volumetric flask, add 50ml of diluent and sonicate for 15 min to dissolve and makeup to the volume (100ml) with dilute and filter through 0.45µ filter.

Accuracy 150% solution:

600 mg of Ibuprofen and 97.5 mg Caffeine sample was weighed and transferred into 100 ml volumetric flask, add 50ml of diluent and sonicate for 15 min to dissolve and makeup to the volume (100m) with dilute and filter through 0.45µ filter.

Acceptance criteria:

The % Recovery for each level should be between 98.0 to 102.0%.

PRECISION:

Method Precision

Method precision was analyzed for Ibuprofen and Caffeine in 6 replicate sample preparations.

Acceptance criteria:

The % RSD for the area of six standard and sample injections results not be more than 2.

INTERMEDIATE PRECISION/ RUGGEDNESS

Intermediate precision was analyzed as part of Method precision in 6 replicates for Ibuprofen and Caffeine in the same lab but by a different Analyst, different column and on a different day.

Acceptance criteria:

The % RSD for the area of six standard and sample injections results not be more than 2.

ROBUSTNESS:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. For this method the robustness was determined by the analysis of the samples under variety of conditions such as: Influence of variations of Organic phase ratio (±2), column Temperature (±5) and Change in wavelength (±0.2).

Acceptance criteria:

The % RSD for the area of six standard and sample injections results not be more than 2.

The % RSD for the area of two sample injections results not be more than 2.

FILTER VALIDATION

The sample solution was divided into three portions. Centrifuge one portion of the sample for 15 min at 2500 rpm in a centrifuge; filter the other portion of sample through 0.45µ nylon filter as per method and filter the third portion through whattman filter No.42.

Acceptance criteria:
The % RSD for the area of six standard results not more than 2.
The % RSD for the area of different filters sample injections results not more than 2.

SOLUTION STABILITY

Stability of standard and sample solution for Ibuprofen and Caffeine was performed by injected standard and sample solution with different time interval from the time of injection. Solutions shall be injected once in 0 hours, 8 hours, 16 hours and 24 hours. The stability of solution was decided based on the area obtained at different time interval. If the results were not within the acceptance criteria, the test was discontinue and reported were solution was to be stable.

Acceptance criteria:
The % RSD for the area of four standard results not more than 2.
The % RSD for the area of different time intervals sample injections results not more than 2.

FORCED DEGRADATION STUDY:

Acid degradation:

About 1020 mg was taken into a reflux flask, added 5ml of 0.1N hydrochloric acid to the flask and refluxed for 30 min at 60 º C. After refluxing, cooled the sample and added same quantity of 0.1N sodium hydroxide so as to neutralize the solution. Transferred contents into a 100 ml volumetric flask. Added 50 ml of diluents and sonicated for 15min, cooled made up the volume with diluents mixed well and filtered through 0.45µm nylon filter.

Base degradation:

About 1020 mg was taken into a reflux flask, added 5ml of 0.1N sodim hydroxide to the flask and refluxed for 30 min at 60 º C. After refluxing, cooled the sample and added same quantity of 0.1N hydrochloric acid so as to neutralize the solution. Transferred contents into a 100 ml volumetric flask. Added 50 ml of diluents and sonicated for 15 min, cooled made up the volume with diluent mixed well and filter through 0.45µm nylon filter.

Peroxide degradation:

About 1020 mg was taken into a reflux flask, added 5 ml of 1% hydrogen peroxide to the flask and refluxed for 30 min at 60 º C. After refluxing, cooled the sample and transferred contents into a 100ml volumetric flask. Added 50 ml of diluent and sonicated for 15 min, cooled made up the volume with diluent mixed well and filtered through 0.45µm nylon filter.

Heat degradation:

About 1020 mg of medicament and placebo exposed to heat 30 min at 105 º C. Sample was prepared by taking 1020 mg into a 100ml volumetric flask. Added 50ml of diluent and sonicated for 15min cooled, then made up the volume with diluent mixed well and filtered through 0.45µm nylon filter.

Humidity:

About 1020 mg of medicament and placebo exposed to humidity 90% RH and 25ºc in a desicator for 7 days. Sample was prepared by taking 1 capsule into a 100ml volumetric flask. Added 50ml of diluent and sonicated for 15min, cooled made up the volume with diluent mixed well and filtered through 0.45µm nylon filter.

Water hydrolysis:

About 1020 mg was taken into a reflux flask, added 5 ml of water to the flask and refluxed for 30 min at 60º C. After refluxing, cooled the sample and transferred contents into a 100 ml volumetric flask. Added 50 ml of diluent and sonicated for 20 min cooled then made up the volume with diluent mixed well and filtered through 0.45µm nylon filter

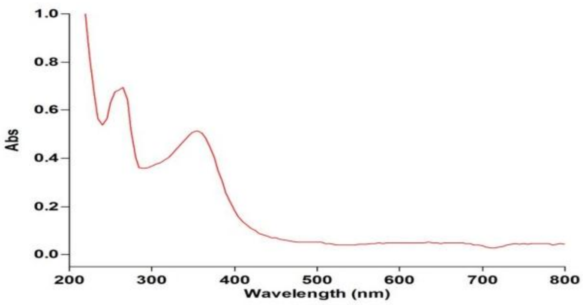
V. RESULTS AND DISCUSSION

Melting point

The melting point for the Ibuprofen and Caffeine was found to be 76^{o}C and 235^{o}C. Hence the selected drug is pure and stable.

Wavelength detection

100µg/ml solution of Ibuprofen and Caffeine was scanned at UV region from 200-800nm. The wavelength detected was found to be 254nm.



Solubility studies

Table no 6: Solubility studies of Ibuprofen and Caffeine

S.No	Solvent	Drug	%Solubility
1	Methanol	Ibuprofen	(100mg/ml)
		Caffeine	(85mg/ml)
2	Water	Ibuprofen	Insoluble
		Caffeine	Insoluble
3	Ethanol	Ibuprofen	(94mg/ml)
		Caffeine	(87mg/ml)
4	Chloroform	Ibuprofen	(84mg/ml)
		Caffeine	(73mg/ml)
5	Acetonitrile	Ibuprofen	(98mg/ml)
		Caffeine	(95mg/ml)

Factorial Design studies

Table no 7: Runs of Caffeine

Run	Factor 1 A:Flow rate mL/min	Factor 2 B: Wave length nm	Factor 3 C: Column temp ^{0}C	Response 1 RT Min	Response 2 Peak area mV	Response 3 Tailing factor
1	0.15	256	4	1.50	15530176	1.2209
2	0.15	252	4.5	1.47	15765754	1.2941
3	0.1	252	4.5	1.58	15685460	1.3293
4	0.05	254	4.5	1.57	15543175	1.3189
5	0.05	252	4	1.63	15565507	1.4004
6	0.05	256	4.5	1.61	15294924	1.2779
7	0.15	252	4	1.57	15879257	1.3269
8	0.1	254	5	1.57	15515038	1.2643
9	0.1	256	5	1.57	15249890	1.2780
10	0.1	252	5	1.61	15646899	1.2284
11	0.1	256	4.5	1.60	15333381	1.2943
12	0.1	256	4	1.68	15374755	1.2620
13	0.15	254	5	1.40	15470243	1.2535
14	0.15	254	4	1.45	15699127	1.2870
15	0.05	256	5	1.63	15298467	1.2432
16	0.05	252	4.5	1.61	15695332	1.3258
17	0.05	256	4	1.69	15214814	1.2668
18	0.15	256	4.5	1.39	15369154	1.2723
19	0.15	252	5	1.44	15601467	1.2099
20	0.05	254	5	1.61	15546506	1.2411
21	0.15	254	4.5	1.41	15540584	1.3012
22	0.1	254	4	1.64	15570523	1.3280
23	**0.1**	**254**	**4.5**	**1.59**	**15549475**	**1.3251**

24	0.15	256	5	1.42	15200726	1.2735
25	0.05	252	5	1.65	15838808	1.2095
26	0.05	254	4	1.65	15407157	1.3404
27	0.1	252	4	1.66	15781470	1.3704

- Run 23 were selected for analysis of Caffeine

Table no 8: Runs of Ibuprofen

Run	Factor 1 A:Flow rate mL/min	Factor 2 B: Wave Length nm	Factor 3 C: Column Temp ^{0}C	Response1 RT Min	Response2 Peak area mV	Response3 Tailing factor
1	0.15	252	5	6.26805	4152132	1.1899
2	0.05	256	5	6.46181	4233492	1.2854
3	0.05	254	5	6.47903	4152132	1.2916
4	0.05	256	4	6.26465	4233492	1.2937
5	0.15	254	5	6.36515	4358652	1.2231
6	0.1	256	4	6.34662	4141378	1.2581
7	0.15	254	4	6.5001	4183132	1.2872
8	0.05	252	4.5	6.5772	4343452	1.2413
9	0.1	256	4.5	6.50051	4163112	1.2536
10	0.1	256	5	6.44875	4232452	1.2084
11	0.1	254	4	6.48864	4346651	1.2399
12	0.05	254	4	6.42928	4164365	1.2704
13	0.15	256	5	6.39117	4175131	1.2005
14	0.1	252	5	6.3672	4265492	1.1813
15	0.1	254	5	6.44093	4135135	1.2171
16	0.15	252	4.5	6.50898	4235425	1.2284
17	0.1	252	4.5	6.56331	4362653	1.2017
18	0.05	254	4.5	6.55942	4153352	1.2996
19	0.15	256	4.5	6.50055	4142132	1.2666
20	0.05	252	4	6.51814	4362542	1.1992
21	0.15	256	4	6.40391	4152341	1.2886
22	0.15	252	4	6.55032	4263251	1.2136
23	0.15	254	4.5	6.54583	4332514	1.2741
24	0.1	252	4	6.55002	4162534	1.1682
25	0.05	252	5	6.42981	4251352	1.2403
26	**0.1**	**254**	**4.5**	**6.57097**	**4325135**	**1.2536**
27	0.05	256	4.5	6.47138	4152135	1.3118

- Run 26 were selected for analysis of Ibuprofen.

Table no 9: Responses of RT, Peak area, tailing Factor of Caffeine.

Responses Source	Retention time			Peak area			Tailing Factor			Significant/ Non-Significant
	Sum of Squares	Df	p-value	Sum of Squares	df	p-value	Sum of Squares	Df	p-value	
Model	0.2156	9	< 0.0001	9.220E+11	9	<0.0001	0.0591	9	< 0.0021	Significant
A-Flow rate	0.1452	1	< 0.0001	2.360E+10	1	0.0002	0.0019	1	< 0.0321	Significant
B-Wave Leangth	0.0010	1	0.12901	7.175E+11	1	<0.0001	0.0054	1	<0.1282	Significant
C-Column Temp	0.0176	1	< 0.0001	2.382E+10	1	0.0002	0.0206	1	< 0.0001	Significant
AB	0.0040	1	0.00511	1.753E+09	1	0.2249	0.0006	1	< 0.0065	Significant
AC	0.0023	1	0.0252	1.479E+11	1	<0.0001	0.0039	1	< 0.0235	Significant

BC	0.0008	1	0.17130	4.470E+09	1	0.0604	0.0198	1	< 0.0231	Significant
A^2	0.0310	1	< 0.0001	4.160E+07	1	0.2485	0.0015	1	< 0.0001	Significant
B^2	0.0052	1	0.0018	2.366E+09	1	0.1616	0.0011	1	< 0.0016	Significant
C^2	0.0086	1	0.0002	4.959E+08	1	0.4119	0.0043	1	< 0.0012	Significant
Residual	0.0065	17		1.878E+10	17		0.0001	17		
Cor Total	0.2221	26		9.407E+11	26		0.0592	26		

- ➢ The p value for the responses of Retention time, peak area and Tailing factor were to be < 0.500 so all the responses of Caffeine are significant.

Table no 10: Responses of RT, Peak area, tailing Factor of Ibuprofen

Responses Source	**Retention time**			**Peak area**			**Tailing Factor**			**Significant/ Non-Significant**
	Sum of Squares	**df**	**p-value**	**Sum of Squares**	**df**	**p-value**	**Sum of Squares**	**Df**	**p-value**	
Model	0.1975	9	<0.0251	3.180E+13	9	<0.0001	0.0423	9	<0.0235	significant
A-Flow Rate	0.0014	1	<0.0001	2.448E+12	1	<0.0521	0.0038	1	<0.0421	Significant
B-Wave Length	0.0164	1	<0.0012	1.326E+12	1	<0.0013	0.0140	1	<0.0001	Significant
C-Column Temp	0.0089	1	<0.0211	6.405E+11	1	<0.0023	0.0018	1	<0.0012	Significant
AB	0.0073	1	<0.3242	1.535E+12	1	<0.0234	0.0006	1	<0.0023	Significant
AC	0.0289	1	<0.0621	4.688E+10	1	<0.0214	0.0044	1	<0.0003	Significant
BC	0.0588	1	<0.2513	5.769E+12	1	<0.0231	0.0026	1	<0.0012	Significant
A^2	0.0020	1	<0.0243	1.074E+13	1	<0.0151	0.0076	1	<0.0005	Significant
B^2	0.0075	1	<0.0005	5.981E+12	1	<0.0234	0.0043	1	<0.0016	Significant
C^2	0.0663	1	<0.0012	3.319E+12	1	<0.0236	0.0030	1	<0.0021	Significant
Residual	0.0002	17		2.563E+10	17		0.0002	17		
Cor Total	0.1977	26		3.183E+13	26		0.0424	26		

- ➢ The p value for the responses of Retention time, peak area and Tailing factor were to be < 0.500 so all the responses of Ibuprofen are significant.

Table no 11: Predicted and observed results of Ibuprofen and Caffeine

Responses	**Compound**	**Predicted results**	**Observed results**
Retention time (min)	Caffeine	1.666	1.9
	Ibuprofen	6.692	6.8
Peak area (mV)	Caffeine	15296438	15789895
	Ibuprofen	4393295	4316741
USP Tailing	Caffeine	1.288	1.4
	Ibuprofen	1.220	1.3
Conditions		Predicted results	Observed results
Flow rate (ml/min)	Caffeine	0.062	0.1
	Ibuprofen	0.115	
Wavelength (nm)	Caffeine	255.661	254
	Ibuprofen	254.876	
Column Temperature (^{o}C)	Caffeine	39.892	40
	Ibuprofen	38.563	

➢ The factor in table 11 are processed with (+) and (-) Deviation were the conditions are tabulated. In table 11 the predicted results are noted down from the point prediction table

PERTURBATION PLOT OF CAFFINE

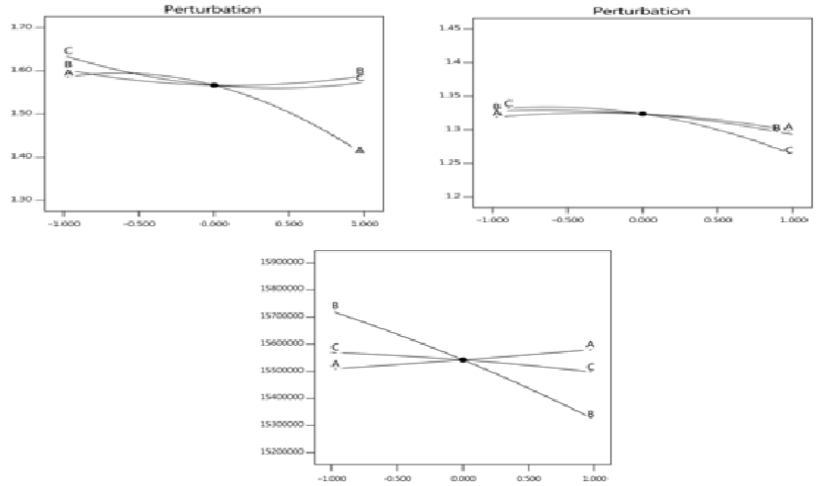


Fig no 2: Pertubation plot of Caffine

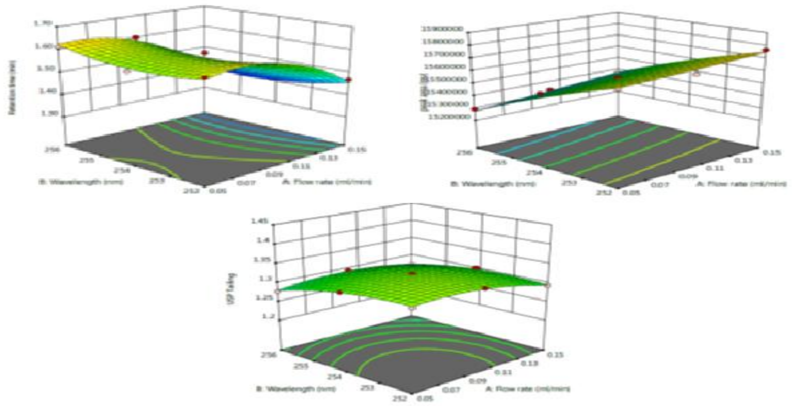


Fig no 3: The 3D surface response plot of Caffeine for optimization of factors

The polynomial equations for the response factors of Caffeine were calculated and given below:

- **Retention time (R_1)** = +468.27593 + 52.59285A -3.70210B + 0.677828C - 0.181684AB – 0.554443AC – 0.008067BC – 28.73072A^2 + 0.0073B^2 + 0.15155C^2.
- **Peak area (R_2)** = - 3.00829 – 1.01960A + 2.49673B + 5.60093C + 1.20850AB – 4.44142AC – 19300BC + 1.05323A^2 – 4964.14443B^2 – 36365.46156C^2
- **Tailing Factor (R_3)** =- 166.62071 – 19.87708A + 1.50861B – 9.50641C + 0.069624AB + 0.719816AC + 0.040660BC – 6.25906A^2 – 0.003361B^2 – 0.106755C^2.

Where R_1, R_2 and R_3 are the response factors i.e. retention time, peak area and number of tailing Factor, respectively and A, B and C are the flow rate, wavelength and column temperature, respectively.

The quadratic effect of flow rate and wavelength separately as well as in interaction was most significant ($p < 0.0142$ and $p < 0.0256$, respectively) on retention time; the quadratic effect of column temperature was also most significant effect ($p < 0.0001$) on peak area whereas the quadratic effect of flow rate and wavelength individually was significant (with $p = 0.0009$ and $p = 0.0041$, respectively) on the number of tailing factor.

Perturbation plot of Ibuprofen

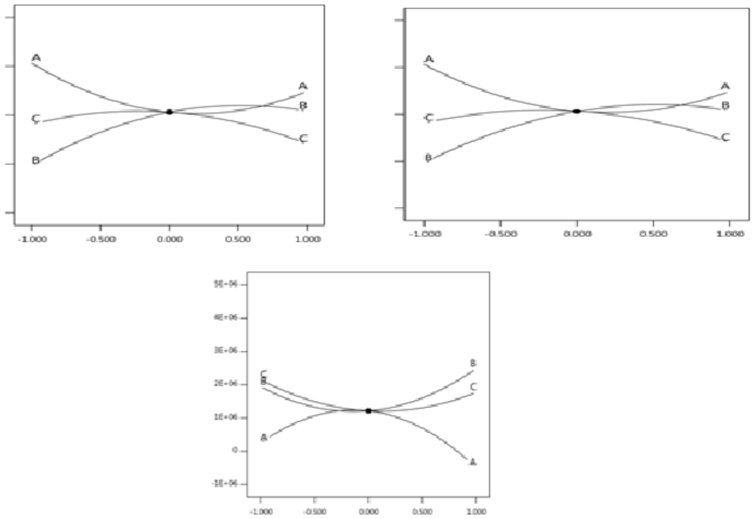


Fig no 4: Pertubation plot of Ibuprofen

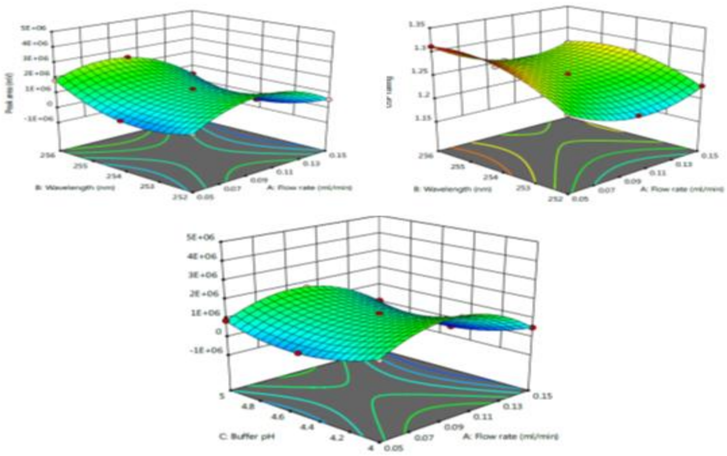


Fig no 5: The 3D surface response plot of Ibuprofen for optimization of factors

The polynomial equations for the response factors of Caffeine were calculated and given below:

- **Retention time (R_1) = -** $482.76937 - 52.45429A + 4.13490B - 13.84235C + 0.246343AB - 1.96179AC + 0.069998BC - 0.008838A^2 - 0.008838B^2 - 0.420607C^2$
- **Peak area (R_2)** $= + 1.52437 + 1.01941A - 1.23190B + 1.49214C - 3.57682AB - 2.50019AC - 6.93366BC - 5.35089A^2 + 2.49614B^2 + 2.97490C^2$.
- **Tailing factor (R_3)** $= -455.68532 + 18.56153A + 3.49555B + 4.60538C - 0.071878AB - 0.766125AC - 0.014721BC + 14.26298A^2 - 0.006709B^2 - 0.089976C^2$.

  Where R_1, R_2 and R_3 are the response factors i.e. retention time, peak area and number of tailing factor, respectively. The A, B and C are the flow rate, wavelength and column temperature, respectively.

The quadratic effect of flow rate and wavelength separately as well as in interaction was most significant ($p < 0.0102$ and $p < 0.0006$, respectively) on retention time; the quadratic effect of column temperature was also most significant effect ($p < 0.0001$) on peak area whereas the quadratic effect of flow rate and wavelength individually was significant (with $p = 0.0001$ and $p = 0.0001$, respectively) on the number of theoretical plates.

Similarly, the Retention time, peak area, and number of theoretical plates of each injection were entered in Design Expert_version 11 software and analysed using the ANOVA with its significance method. For an experimental design with three variable factors, the suitable model fitting to the data was the quadratic model.

Observation:
Effect of independent variables on response parameters

- **Effect of independent variable on Retention time:**

When flow rate increases retention time decreases. Whereas retention time is influenced due to the change of the flow rate and also the wavelength.
Conclusion: retention time was not only effected by flow rate but also by column temperature. The positive regression coefficient was observed by considering both retention time and column temperature. A 3^3 factorial design was established and polynomial equation was generated by software.

- **Effect of Peak area:**

Effect of independent variable on peak area was departed in table. The positive regression coefficient of wavelength indicates the variation in peak area simultaneously. Flow rate had shown less effect on peak area. From design it clearly indicates wavelength had shown little effect on peak area. While considering both wave length and column temperature the increment of peak area was observed.

- **Effect of Tailing factor:**

Effect of independent variable on tailing factor was departed in table. The positive regression coefficient of wavelength indicates the increment of tailing factor simultaneously. Flow rate had shown moderate effect on tailing factor. From design it clearly indicates column temperature had shown little effect on tailing factor. While considering both wave length and column temperature the increment of tailing factor was observed.

TRIAL 1:

Instrument	: UPLC with PDA Detector
Column	: UHP ASB C18, 2.1x50mm, 1.9µm (or equivalent)
Flow rate	: 0.2 ml/min
Column Temperature	: 40°C
Detector	: 254nm
Run time	: 15minutes
Mobile phase	: Methanol: Water (50:50% v/v) (P^H 4.5, adjusted with OPA)
Injection volume	: 1µl

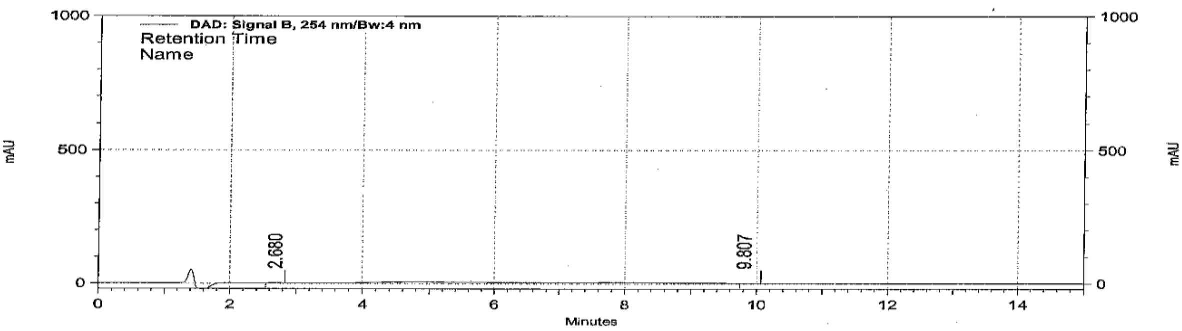


Fig no 6: Chromatogram for Caffeine and Ibuprofen

Observation:
From the above chromatogram peak elution, peak separation and peak shape was not good and the peak response is not good.

TRIAL 2:

Instrument	: UPLC with PDA Detector
Column	: UHP ASB C18, 2.1x50mm, 1.9µm (or equivalent)
Flow rate	: 0.3 ml/min
Column Temperature	: 40°C
Detector	: 254nm
Run time	: 15minutes
Mobile phase	: Methanol: Water (60:40% v/v) (P^H 4.5, adjusted with OPA)
Injection volume	: 1µl

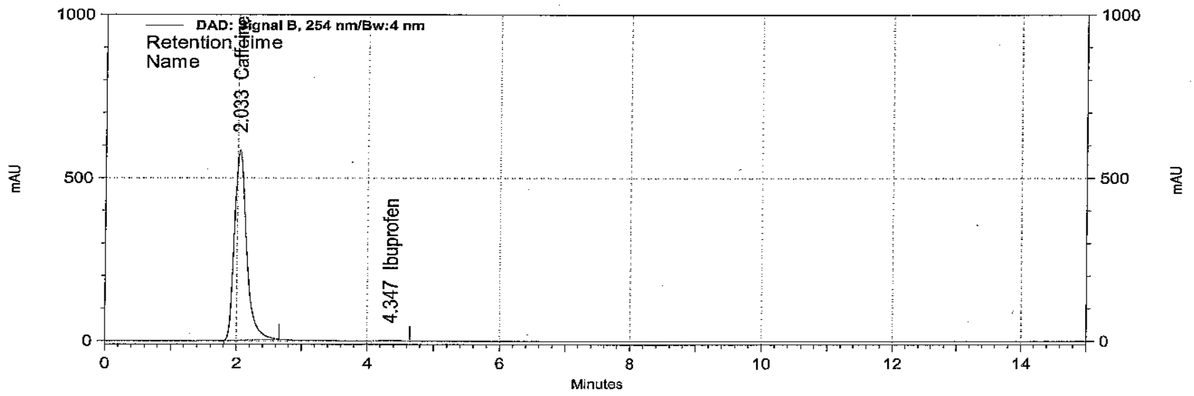


Fig no 7: Chromatogram for Caffeine and Ibuprofen

Observation:
From the above chromatogram it was observed that the Caffeine peak was tailing due to the improper mobile phase selection. The increase in the flow rate concentration the peak was shift to 2 and 4.3 min due to the increase in the pressure. And the chromatograph results showed the elution was completed within 5 min.

TRIAL 3:

Instrument	:	UPLC with PDA Detector
Column	:	UHP ASB C18, 2.1x50mm, 1.9µm (or equivalent)
Flow rate	:	0.1 ml/min
Column Temperature	:	40°C
Detector	:	254nm
Run time	:	15minutes
Mobile phase	:	Methanol: Water (70:30% v/v) (P^H 4.5, adjusted with OPA)
Injection volume	:	1µl

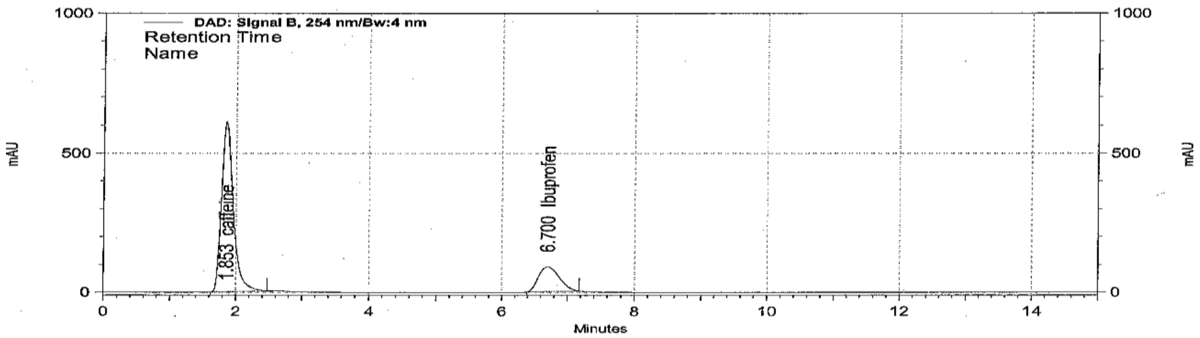


Figure no 8: Chromatogram for Caffeine and Ibuprofen

Observation:
From the above chromatogram it was observed that the Caffeine and Ibuprofen peak showed good separation but the retention time was changing due to the increase of p^H in the mobile phase. The main reason is that the p^H as water keeps a changing.

Optimized Chromatographic Conditions:

Instrument	:	UPLC with PDA Detector
Column	:	UHP ASB C18, 2.1x50mm, 1.9µm (or equivalent)
Flow rate	:	0.1ml/min
Column Temperature	:	40°C
Detector	:	254nm
Run time	:	15minutes
Mobile phase	:	Methanol: Water (70:30% v/v) (P^H 4.5, adjusted with OPA)
Diluent	:	Methanol: Water (70:30 (P^H 4.5, adjusted with OPA)
Injection volume	:	1µl

SYSTEM SUITABILITY:

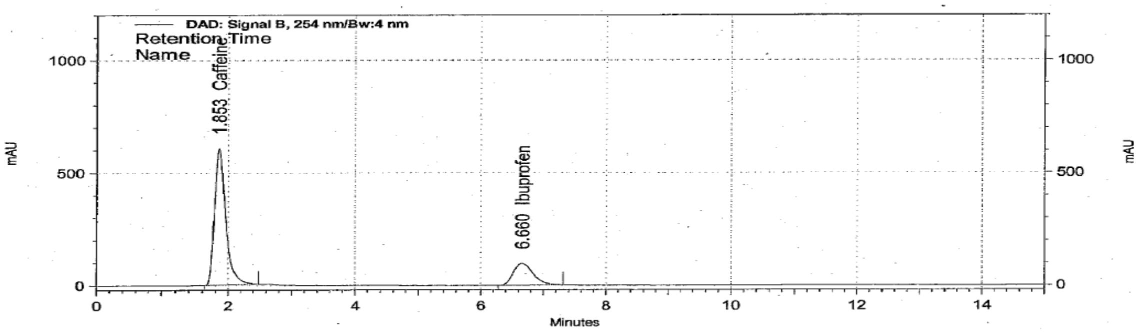


Fig no 9: Chromatogram for system suitability

Good separation was achievement and the retention time was not changing due to the usage of buffer solution.
System Suitability Results:
1). Tailing factor Obtained from the standard injection is 1.3
2). Theoretical Plates Obtained from the standard injection is 43056

FACTORIAL DESIGN:

Table no 13: Calculation of Desirability and Residual values

VALIDATION OF OPTIMIZED FACTORS					
Response	**Compound**	**Predicted results**	**Observed results**	**Residual values (%)**	**Desirability**
Retention time	Caffeine	1.666	1.9	-113.046	1.0000
	Ibuprofen	6.692	6.8	560.320	1.0000
Peak area	Caffeine	15296438	15789895	-3.2200	1.0000
	Ibuprofen	4393295	4316741	1.7425	1.0000
USP Tailing	Caffeine	1.288	1.4	-8.695	1.0000
	Ibuprofen	1.220	1.3	-6.557	1.0000

% Residual values = <u>Predicted results-observed results x 100</u>
Predicted results

VALIDATION PARAMETERS:
SPECIFICITY

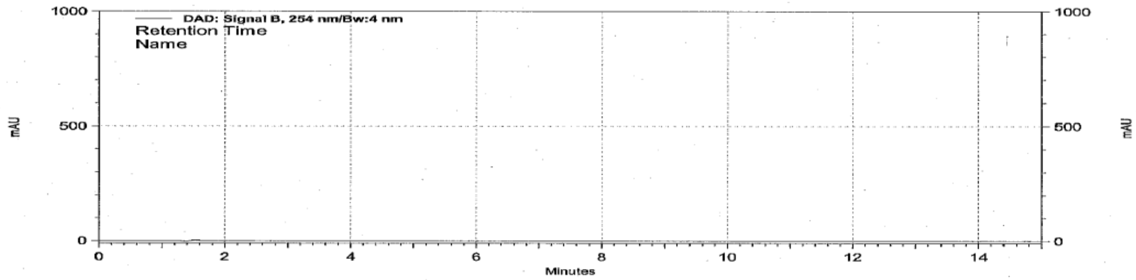


Fig no 10: Chromatogram for blank

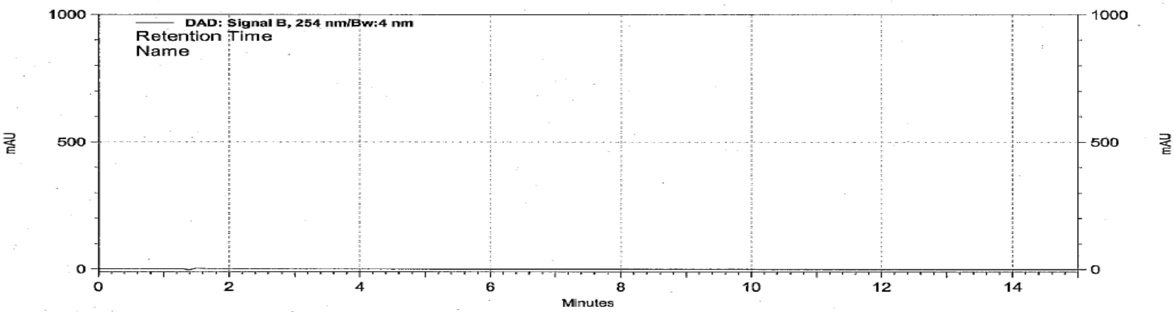


Fig no 11: Chromatogram for placebo

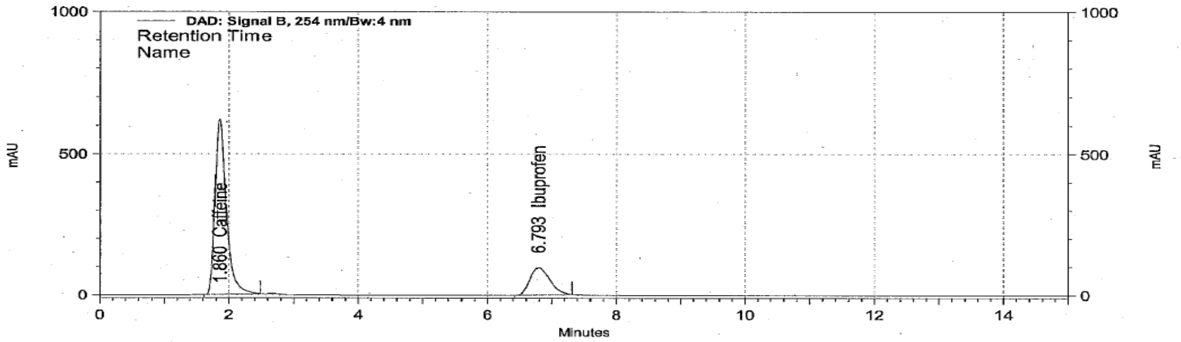


Fig no 12: Chromatogram for Caffeine and Ibuprofen (standard)

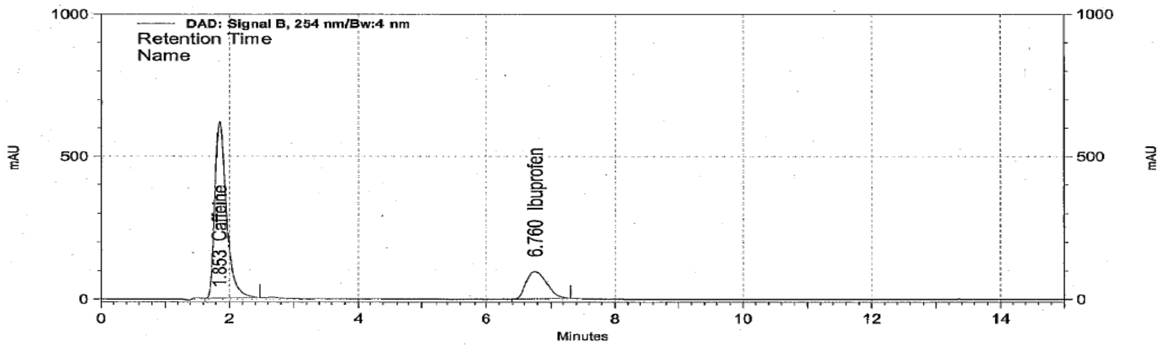


Fig no 13: Chromatogram for Caffeine and Ibuprofen (sample)

Table No 14: Specificity of Ibuprofen and Caffeine

	RT		Area	
Preparation	**Ibuprofen**	**Caffeine**	**Ibuprofen**	**Caffeine**
Blank	NA	NA	NA	NA
Placebo	NA	NA	NA	NA
Standard solution	6.8	1.9	4270361	15820737
Sample solution	6.8	1.9	4316741	15789895

- The sample solution, standard and blank solution are injected. Where the optimized chromatographic conditions and the results shows the method is specific.

LINEARITY AND RANGE:

The linearity of a method is its ability to obtain results that are directly proportional to the sample concentration over a given range. The peak area and concentration were plotted to get a standard calibration curve.

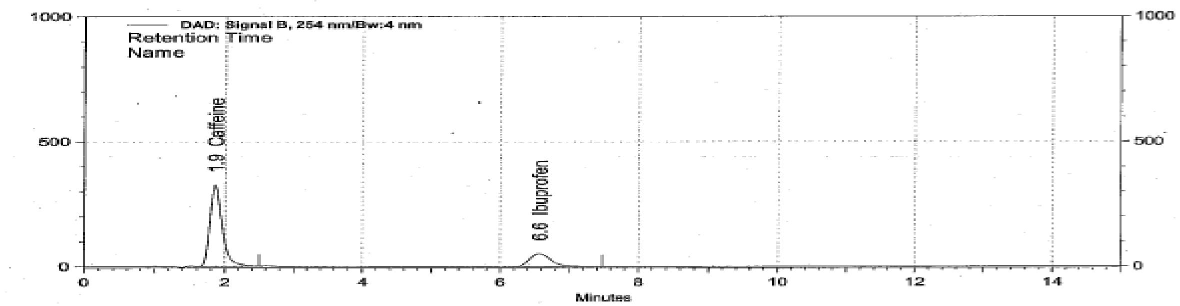


Fig no 14: Chromatogram of 50% linearity

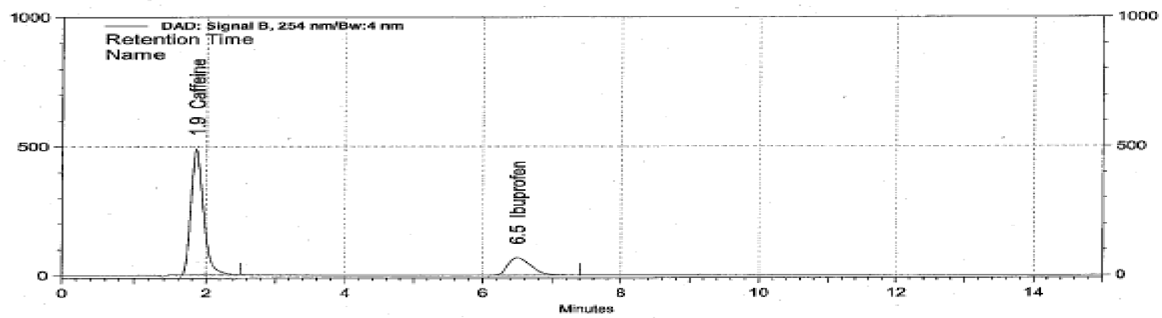


Fig no 15: Chromatogram of 75% linearity

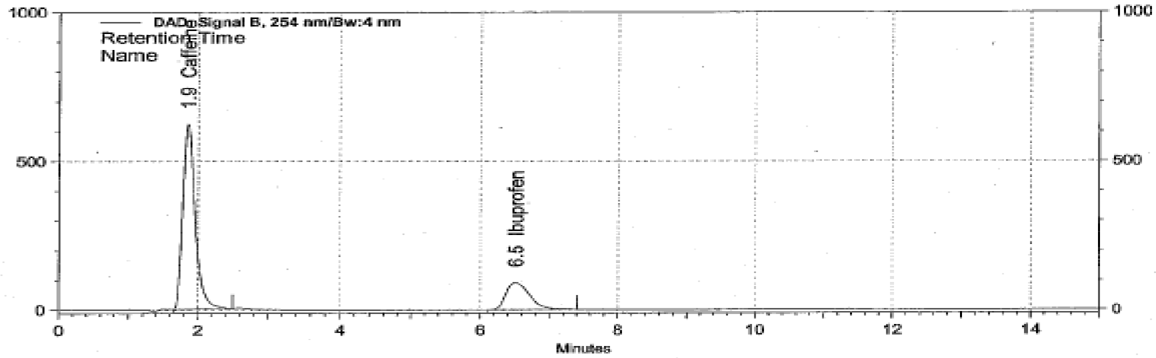


Fig no 16: Chromatogram of 100% linearity

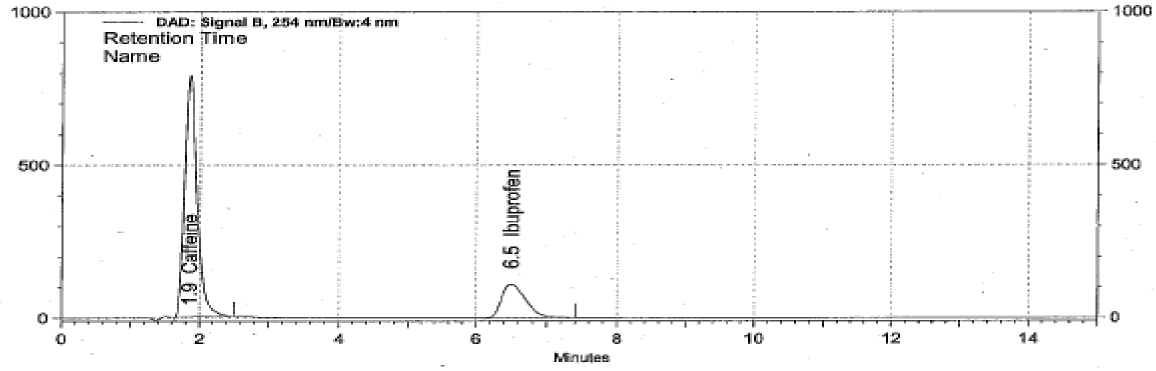


Fig no 17: Chromatogram of 125% linearity

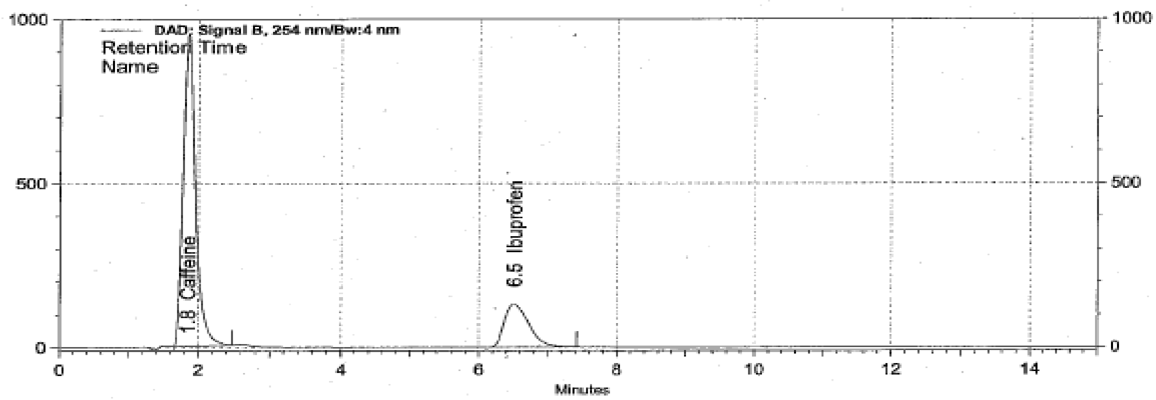


Fig no 18: Chromatogram of 150% linearity

Table No 15: Calibration of Ibuprofen and Caffeine

Level	Conc	Ibuprofen Mean area	Std Dev	Conc	Caffeine Mean Area	Std Dev
50	2001.2	2282754	6842.2	325.2	8284395	41200.1
75	3001.8	3302922	1672.0	487.8	12567843	67466.1
100	4002.4	4590294	15475.1	650.4	16166007	70625.5
125	5003	5466235	7746.0	813	20416749	57514.2
150	6003.6	6713020	29683.4	975.6	24791602	98723.9
	R^2= 0.999			R^2= 0.9996		

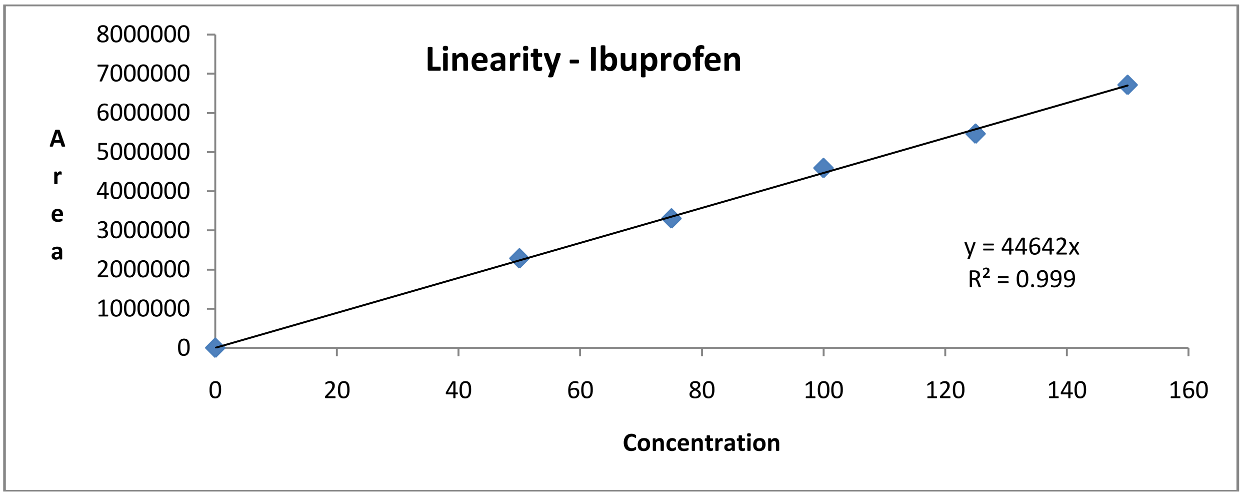


Figure no 19: Linearity graph of Ibuprofen

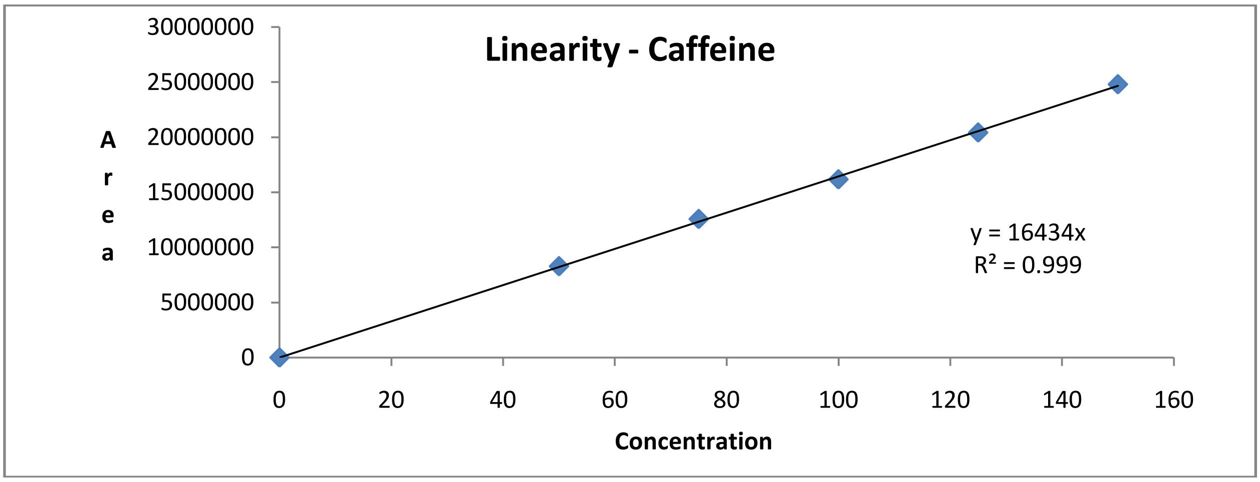


Figure no 20: Linearity graph of Caffeine

Discussion:

The peak area and concentration were plotted at X and Y axis respectively for the different concentration range from 2001.2µg/ml-6003.6µg/ml and 325.2µg/ml-975.6µg/ml. Correlation coefficient value for calibration plot of Ibuprofen was found to be 0.9999 and Caffeine was found to be 0.9996.

ACCURACY

The accuracy of the method is the closeness of the measured value to true value for Ibuprofen and Caffeine sample. Accuracy is usually determined by recovery studies.

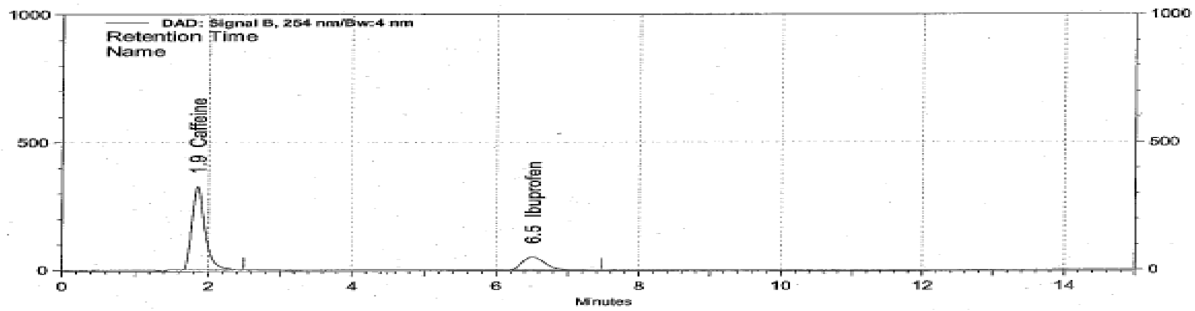


Fig no 21: Chromatogram of 50% accuracy

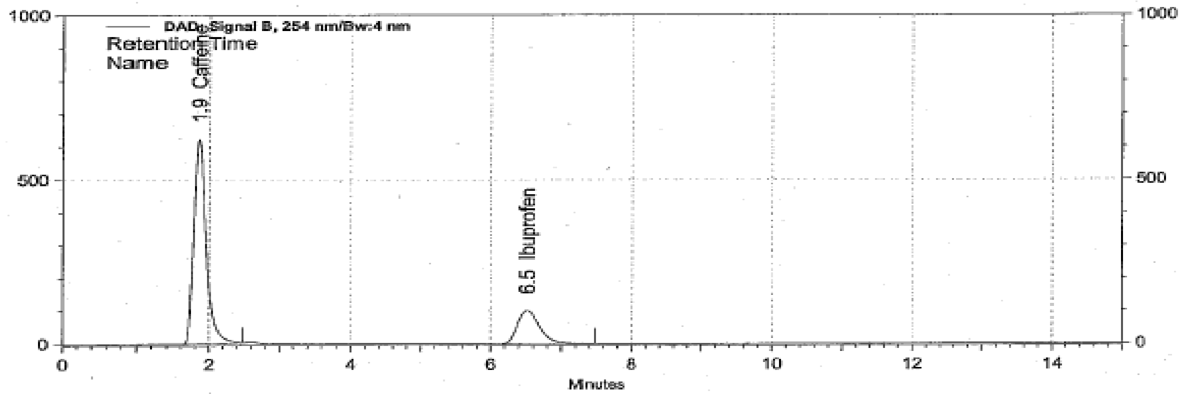


Fig no 22: Chromatogram of 100% accuracy

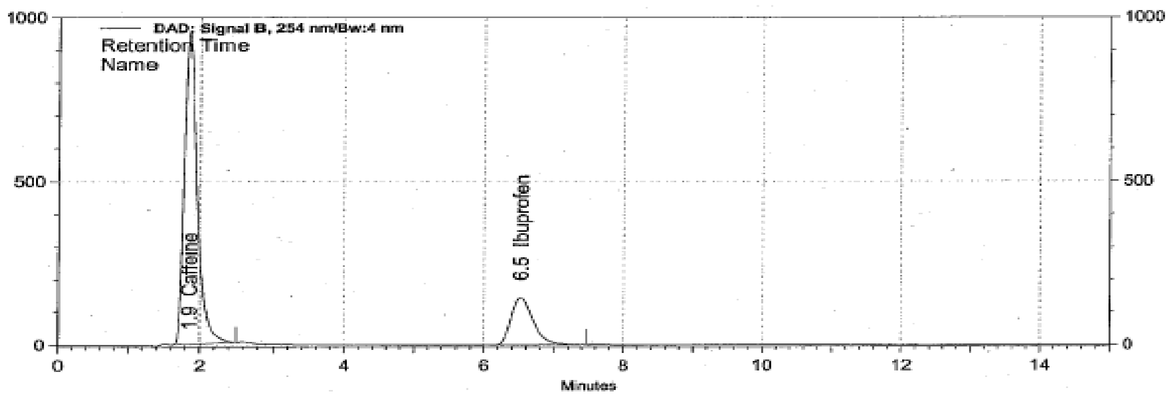


Fig no 23: Chromatogram of 150% accuracy

Table no 16: Accuracy for Ibuprofen

S.No	Concentration	Mean	Std.Dev	%Rsd	% Recovered
1.	ACCURACY-50%	2282399	0.36036	0.4	100.3
2.	ACCURACY-100%	4528563	0.27978	0.3	100.9
3.	ACCURACY-150%	6645572	0.40539	0.4	99.7

Table no 17: Accuracy for Caffeine

S.No	Concentration	Mean	Std.Dev	%Rsd	%Recovered
1.	ACCURACY-50%	8193179	0.36844	0.4	100.6
2.	ACCURACY-100%	15714658	0.13884	0.1	100.0
3.	ACCURACY-150%	24446314	0.34746	0.3	101.1

Discussion:

- The percentage recovery of Ibuprofen was found to be 100.3%, 100.9% and 99.7% for accuracy 50%, 100%, and 150% samples respectively. The %RSD of the samples was found to be 0.4, 0.3 and 0.4.

- The percentage recovery of Caffeine was found to be 100.6%, 100.0% and 101.1% for accuracy 50%, 100%, and 150% samples respectively. The %RSD of the samples was found to be 0.4, 0.1 and 0.3.

PRECISION:

Method Precision

The precision studies were carried out by

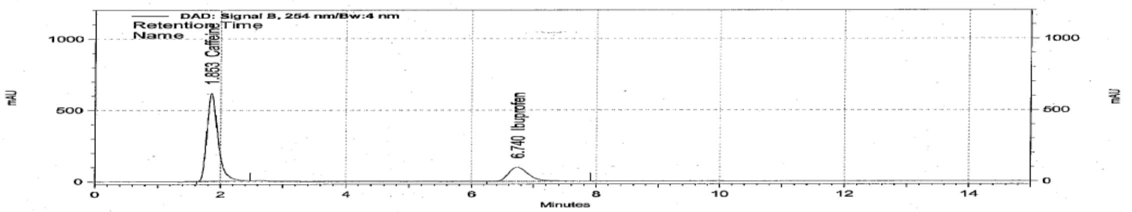


Fig no 24: Chromatogram of standard

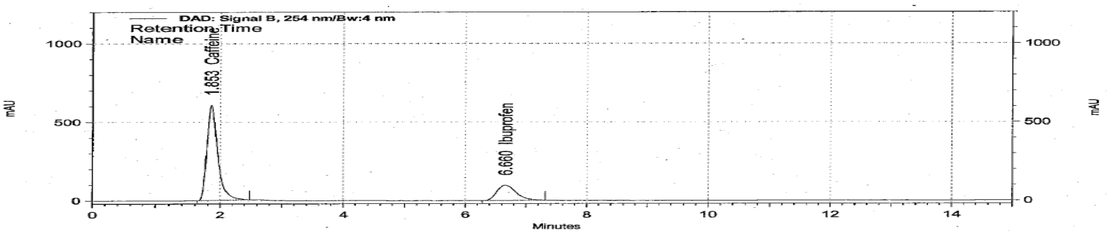


Fig no 25: Chromatogram of sample

Table no 18: System Precision of Ibuprofen

S.No	Peak name	Rt(min)	Area	USP plate count	USP tailing
1	Ibuprofen	6.7	4390188	42837.6	1.3
2	Ibuprofen	6.7	4404772	43303.7	1.3
3	Ibuprofen	6.6	4425712	42872.7	1.3
4	Ibuprofen	6.6	4550264	42614.4	1.4
5	Ibuprofen	6.6	4473053	42854.2	1.3
6	Ibuprofen	6.6	4530214	42536.3	1.4
Mean			4462367		
Std. Dev.			66804		
% RSD			1.4		

Table no 19: System Precision of Caffeine

S.No	Peak name	Rt(min)	Area	USP plate count	USP tailing
1	Caffeine	1.9	15636693	11326.4	1.5
2	Caffeine	1.9	15665017	11492.4	1.5
3	Caffeine	1.8	15773564	1138554	1.5
4	Caffeine	1.9	15656352	11432.1	1.5
5	Caffeine	1.9	15918336	11408.4	1.5
6	Caffeine	1.9	15755788	11493.4	1.5
Mean			15734292		
Std. Dev.			106090.3		
% RSD			0.6		

Discussion:

Her mean, Standard deviation, % RSD was calculated and the results revealed that the % RSD was found to be <2%. Hence the results were within the limits.

The %RSD value indicates a good degree of precision within the specified limits.

Intermediate Precision (RUGGEDNESS):

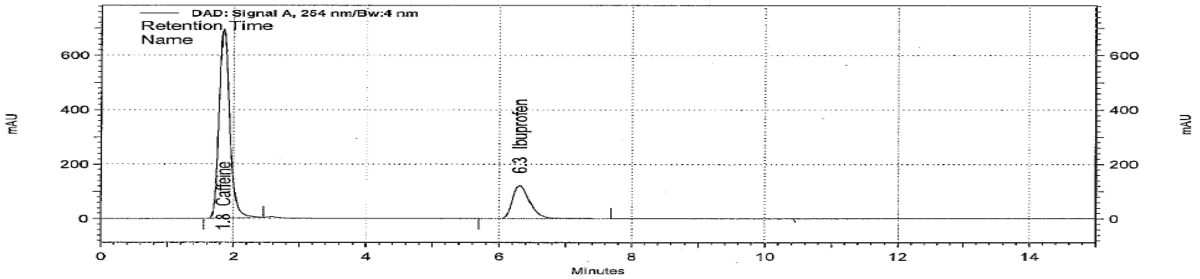


Fig no 26: Chromatogram of standard

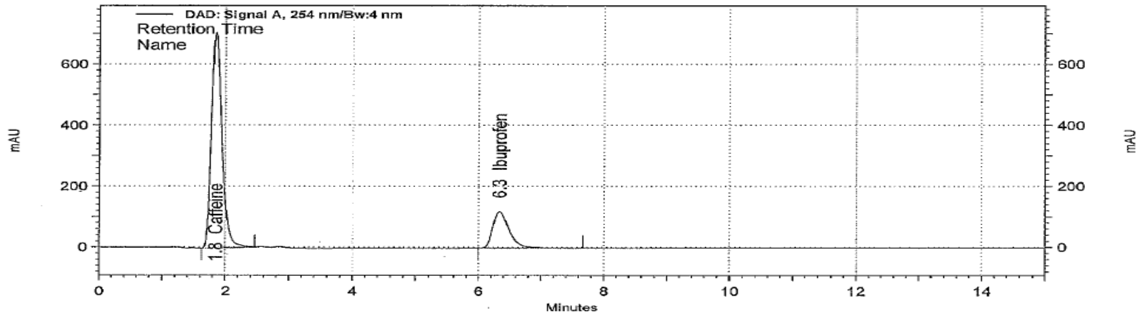


Fig no 27: Chromatogram of sample

Discussion:
Here Intermediate precision /ruggedness was performed with different analyst in same lab and and same day.
ROBUSTNESS:
To establish the robustness of the UPLC method employed for analysis of assay of, the method was challenged for various parameters like change in mobile phase ratio, change in column temperature and change in wavelength.
The observations in different conditions are tabulated below

Table no 20: Results for Robustness of Ibuprofen and Caffeine

Condition	Compound	Mean	Std. Dev	%RSD	%Assay
Organic phase (-2%)	Ibuprofen	4188703	44670.7	1.0	103.9
	Caffeine	16003787	273166.0	1.7	100.8
Organicphase (+2%)	Ibuprofen	4404474	13488.0	0.3	101.0
	Caffeine	15962367	85260.1	0.5	100.1
Column Temperature (-5^{o}c)	Ibuprofen	4430617	40229.0	0.2	100.2
	Caffeine	15839019	73109.1	0.4	99.8
Column Temperature (+5^{o}c)	Ibuprofen	4556200	5237.5	0.1	101.7
	Caffeine	16065138	546221.3	0.3	99.9
wavelength (-5 nm)	Ibuprofen	3908065	39817.1	1.0	101.3
	Caffeine	133384435	271469.3	1.7	100.89
wavelength (+5 nm)	Ibuprofen	4692758	47856.2	1.0	101.8
	Caffeine	17940558	358523.3	1.0	100.8

Discussion:
There is no significant effect on the result by doing small deliberate changes in the system as well as in method parameters.

FILTER VALIDATION

Table no 21: Filter paper varaiability of Ibuprofen and Caffeine

S.No	Type	Compound	Mean	Std Dev	%RSD	%Assay
1.	Centrifuged Sample	Ibuprofen	4459050	49598.5	1.1	99.6
		Caffeine	15794910	279721.3	1.7	102.1
2.	Nylon Filter	Ibuprofen	4497841	8941.3	0.2	100.4
		Caffeine	16043207	3555.3	0.0	103.7
3.	Whatman Filter	Ibuprofen	4521811	31860.1	0.7	100.9
		Caffeine	15882256	109334.3	0.6	102.7

Discussion

It is within the limits and the solution was to be stable. No carryover peaks and interferences were observed.

SOLUTION STABILITY:

Table no 22: Solution Stability of Ibuprofen and Caffeine

S.No	Ibuprofen	% Assay	Caffeine	% Assay
1 Hour	433141	99.7	15715255	101.2
8 Hours	447672	99.8	15695433	100.5
16 Hours	448520	99.5	15599477	102.3
24 hours	450385	99.9	15576825	102.8

Discussion

It is within the limits and the solution was to be stable up to 24Hrs.

FORCED DEGRADATION STUDIES:

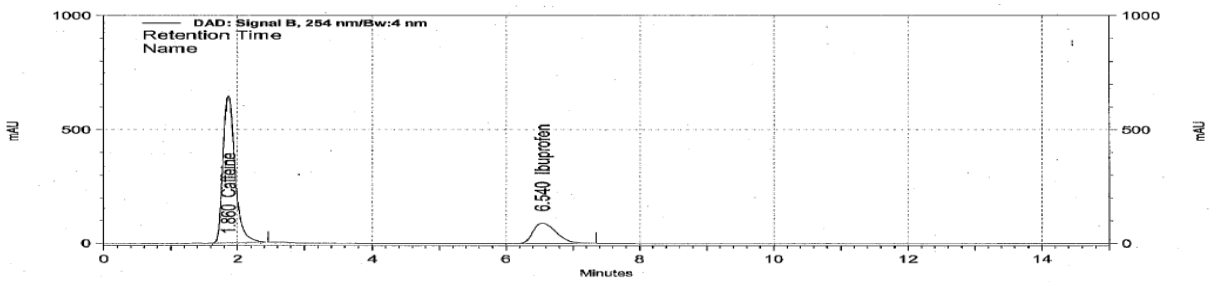


Fig no 28: Chromatogram for Forced Degradation Study

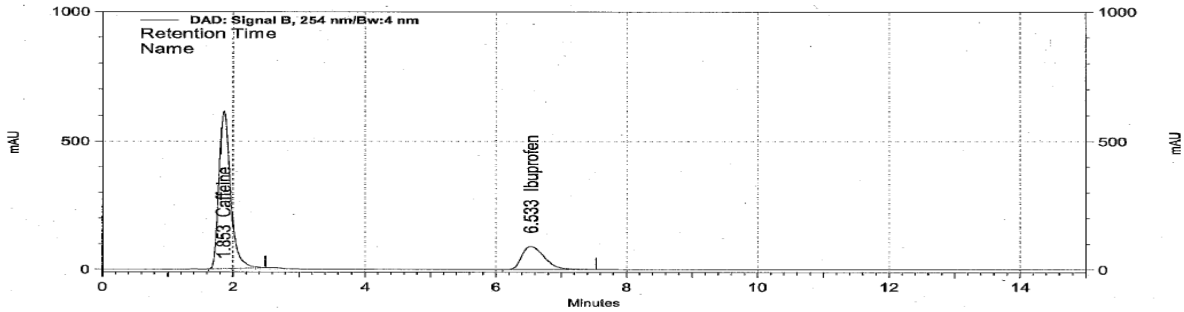


Fig no 29: Chromatogram for Forced Degradation Unstressed Sample

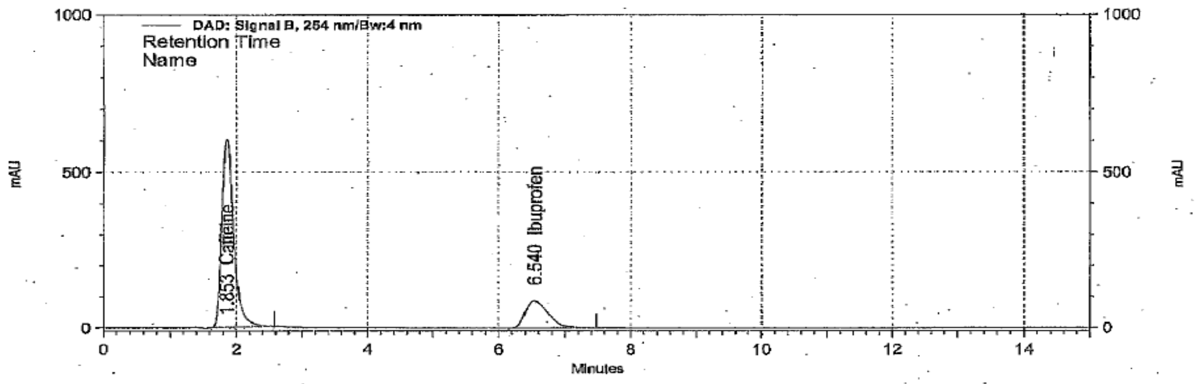


Fig no 30: Chromatogram for Forced Degradation Acid Sample

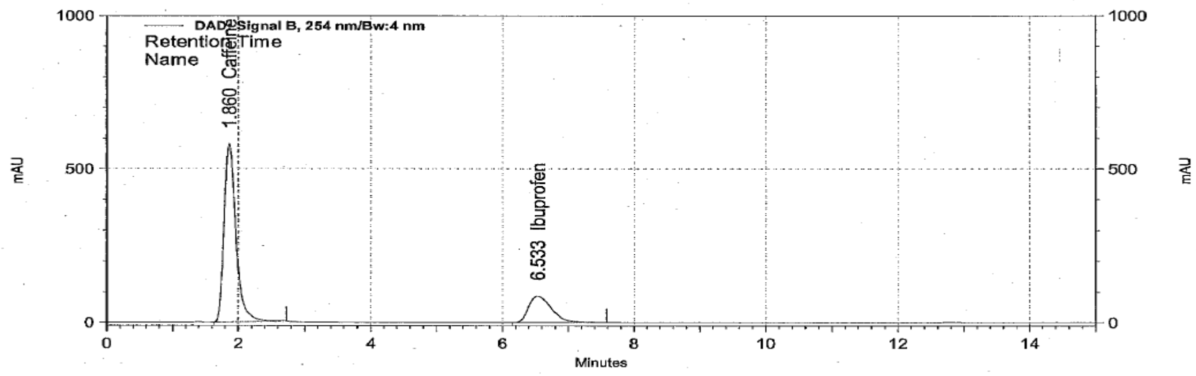


Fig no 31: Chromatogram for Forced Degradation Base/Alkali Sample

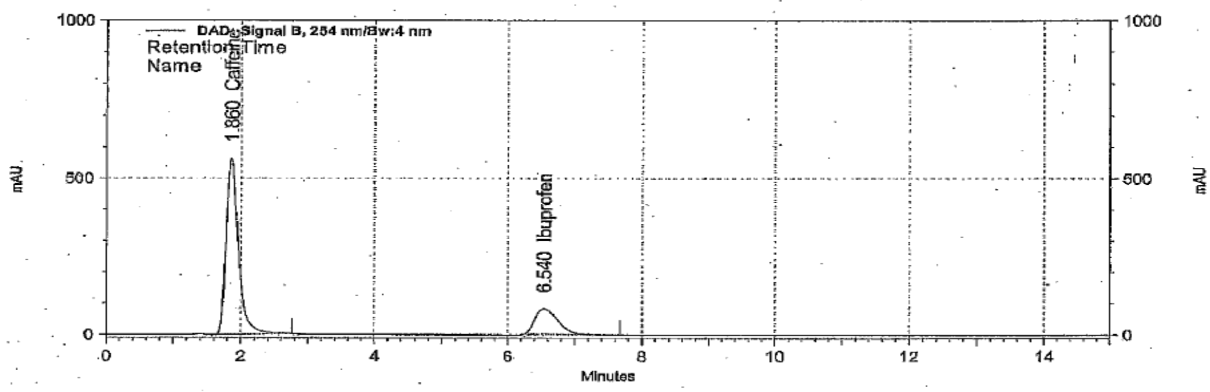


Fig no 32: Chromatogram for Forced Degradation Oxidation Sample

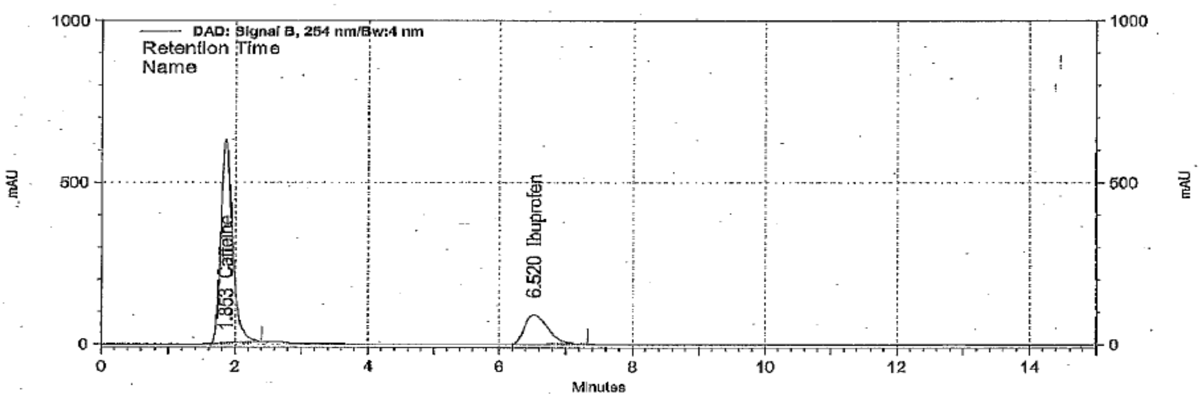


Fig no 33: Chromatogram for Forced Degradation Water Sample

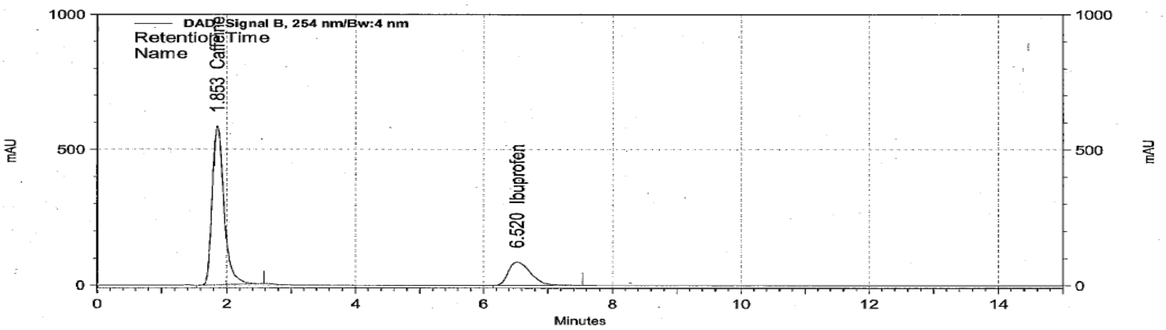


Fig no 34: Chromatogram for Forced Degradation Heat Sample

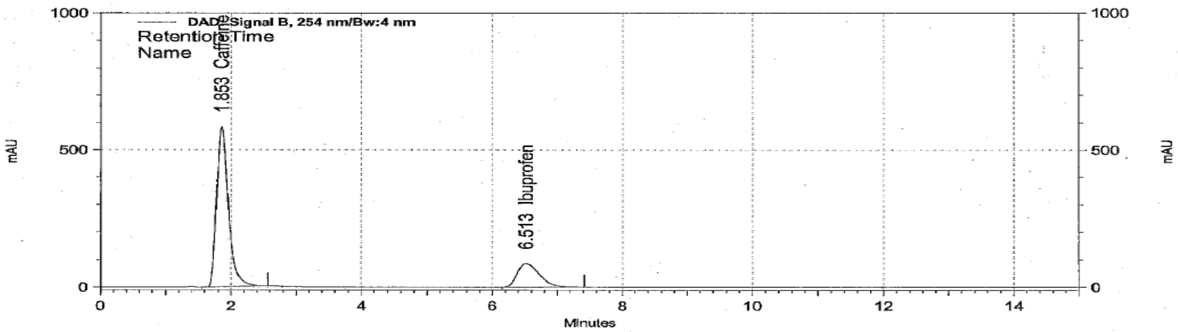


Fig no 35: Chromatogram for Forced Degradation Humidity Sample

Table no 23: Forced Degradation of Ibuprofen

Stressed conditions	**Content in %**	**Ibuprofen**	
		% Degradation	**Peak Purity Index**
Unstressed sample	104.5	NA	NA
Acid hydrolysis(0.1M HCl)	99.3	5.0	1.00
Base hydrolysis (0.1M NaOH)	99.2	5.1	1.00
Oxidation reflux (1%H_2O_2)	100.6	5.9	1.00
Water hydrolysis	99.5	4.8	1.00
heat at 105°c	99.5	4.8	1.00
Humidity 90% RH and 25°	98.5	5.7	1.00

Table no24: Forced degration of Caffeine

Stressed conditions	**Content in %**	**Caffeine**	
		% Degradation	**Peak Purity Index**
Unstressed sample	99.2	NA	NA
Acid hydrolysis (0.1M HCl)	98.0	1.2	1.00
Base hydrolysis (0.1M NaOH)	96.5	2.7	1.00
Oxidation reflux (1%H_2O_2)	98.2	1.0	1.00
water hydrolysis	97.7	1.5	1.00
heat 105°c	97.4	1.8	1.00
humidity 90% RH and 25°c	96.1	3.1	1.00

Discussion

The degradation studies were performed where the acid, alkaline, peroxide, thermal, photolytic and humidity stress conditions were observed. Hence the highest degradation was observed in Ibuprofen at oxidation condition and lowest degradation was observed at oxidation condition.

VI. CONCLUSION

The method was successfully developed and optimized through Design of Experiments, and data was analyzed using Design Expert version 11 software. The significant effect of independent factors was analyzed using ANOVA, and the effect was also reported in the form of perturbation plots. The design of experiments provides efficient tools for the optimization of variable factors for UPLC method development. Further the method was validated and as per the ICH guidelines. The results revealed that the present method is simple, accurate, precise, rapid, economic and robust for the analysis of Ibuprofen and Caffeine in combined dosage form.

The developed method gives an idea for research and development in method development that the factorial design can be applicable successfully for the method development and validation of Ibuprofen and Caffeine, which results in the decreasing the cost, time and manpower. Hence DOE can play a vital role in the method development and validation in future as a powerful analytical tool.

BIBLIOGRAPHY

[1]. Beckett A.H and Stenlake J.B; Text book of pharmaceutical chemistry 4th Edn,-part 2 CBS publishers and Distriburots, New Delhi,1998:278,307

[2]. Douglas Skoog A., James Hollar F. and Timothy Nieman, a Principle of Instrumental Analysis. 5thed., Thomson Learning Inc. Singapore, 1998;110 ,300

[3]. Sharma,B.K., Instrumental method of Chemical Analysis, 24th ed., GOEL Publishing House, Meerut,2005; 46, 68.

[4]. www.Inova.fo/upload/myndasavan/uflc%20 prominence.pdf.

[5]. Kofman J., Zhao Y., Maloney T., Baumgartner T., Bujalski R.: Am. Pharm. Rev. 2006; 9: 88.

[6]. Zhang Y., Gong X., Zhang H., Larock R.C., Yeung E.S.: J. Comb. Chem. 2000; 2: 450.

[7]. Zhou C., Jin Y., Kenseth J.R, Stella M., Wehmeyer K.R, Heineman W.R.: J. Pharm. Sci. 2005; 94: 576.

[8]. Deemter J.J.V., Zuiderweg F.J., Klinkenberg A.: Chem. Eng. Sci. 1956; 5: 271.

[9]. Zhu C., Goodall D., Wren S.: LCGC 2005; 23: 54. Jerkovich A.D., Mellors J.S., and Jorgenson J.W., *LCGC* 2003; 21(7): 660–611. Wu N., Lippert J.A., and Lee M.L., *J. Chromotogr*. 2001; 911(1).

[10]. Michael E Swartz, Ultra performance liquid chromatography UPLC: an introduction. Separation science redefined. 2005; 1: 8-14.

[11]. Munson J.W:Modern Methods of Pharmaceutical Analysis,Medical book distributors,Mumbai,2001,17-54.

[12]. Synder K.L,Kriklad J.J and Glajch J.L:Practical HPLC Method Development 2nd Edn,Wiley-Interscience Publication, USA, 1983,1-10.

[13]. USFDA, Pharmaceutical cgmp for the 21st century − a risk−based approach, in: Second Progress Report and Implementation Plan Introduction, Food and drug Administration, Rockville, MD, 2003.

[14]. Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L.Massart, and Guidance for robustness: ruggedness tests in method validation, J.Pharm. Biomed. Anal. 24 (2001) 723–753.

[15]. P. Sibinovi´c, A.ˇSmelcerovi´c, R. Pali´c, S. Đor–devi´c, V. Marinkovi´c, Ruggedness testing of an HPLC method for the determination of ciprofloxacin, J. Serb. Hem. Soc. 70 (2005) 979–986.

[16]. G.E.P. Box, J.S. Hunter, W.G. Hunter, Statistics for Experimenters: Design, Innovation, and Discovery, Wiley−Interscience, Hoboken NJ, 2005.

[17]. R.E. Bruns, I.S. Scarminio, N.B.d. Barros, Statistical Design − Chemometrics, Elsevier, Amsterdam, 2006.

[18]. Roy, R.K., Design of experiments using the Taguchi Approach. *Wiley-Interscience Publication* 2001, John Wiley & Sons, Inc.

[19]. USFDA, Guidance for Industry: PAT − A Framework for innovative pharmaceutical Development, Manufacturing, and Quality Assurance, Food and Drug Administration, Rockville MD, 2004.

[20]. B. Dejaegher, Y. Vander Heyden, Experimental designs and their recentadvances in setup, data interpretation, and analytical applications, J.Pharm. Biomed. Anal. 56 (2011) 141158.

[21]. B. Dejaegher, Y. Vander Heyden, The use of experimental design inseparation science, Acta Chromatogr. 21 (2009) 161–201.

[22]. B. Dejaegher, Y. Vander Heyden, Robustness tests of CE methods, in: S.J.M.Ahuja (Ed.), Capillary Electrophoresis Methods for Pharmaceutical Analysis,Elsevier, Academic Press, Amsterdam, 2008, pp. 185–224.

[23]. B. Dejaegher, Y. Vander Heyden, Ruggedness and robustness testing, J.Chromatogr. A 1158 (2007) 138–157.