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Simultaneous Quantification of Acetaminophen, Caffeine, and Ibuprofen in Fixed Dose Combination Drug Using HPLC with UV Detection

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Authors' contributions

This work was carried out in collaboration between all authors. Author EKD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors CKA and PSL managed the analyses of the study. Authors DAOA and JSKA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Combination therapy of analgesics is well suited for pain management especially in elderly patients and, has been recommended by the World Health Organization (WHO). Drug analysis plays an important role in the development, manufacture and therapeutic use of drug. In this study, a suitable, cost effective Isocratic HPLC-UV method (Reversed Phase) has been developed and validated for the simultaneous quantification of Acetaminophen, Caffeine, and Ibuprofen in fixed dose combination drugs, using a mobile phase combination of methanol and 0.025M Phosphate buffer –(adjusted to pH 3.2 with Orthophosphoric acid) in the ratio 85:15.A Vertex Column-Eurospher C_{18} (250 x 4.6 mm), flow rate of 1.0 ml/min at 25°C were the chromatographic conditions.

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With Piroxicam as internal standard, quantification was achieved with UV detection at 225 nm based on the peak area responses. A good resolution and a short run time (7mins) were achieved with the validated conditions. In consequence, statistical evaluation at the 95% confidence limits revealed that, the method was Linear; with an average correlation coefficient (R = 0.995), and accurate - (mean recovery 99.45% for Acetaminophen, 100.10% for Caffeine and 99.28% for Ibuprofen). With an instrument and intermediate precision RSDs>2.0, the method was found to be specific, Robust, and more economical. Six formulated combination products on the Ghana market were assayed using the validated method. The Acetaminophen, Caffeine and Ibuprofen contents in the combination drugs varied from 97.35% to 103.88%.

Keywords: Acetaminophen; caffeine; ibuprofen; fixed dose combination analgesic; reversed phase-HPLC; methanol-phosphate buffer; short run time; postoperative.

1. INTRODUCTION

Pain is a prevalent symptom experienced by at least 30% of patients undergoing an oncological treatment for metastatic disease and by more than 70% of advanced cancer patients [1]. In 1986 the World Health Organization [2] published a set of guidelines for cancer pain management based on the three step analgesic ladder [2]. Acute or chronic pain relief can be achieved by multifarious methods, with drug use – analgesic, being the mainstay of treatment. However, no single analgesic is perfect and not all pain yields to classic analgesics. Combination therapy of analgesics is well suited for pain management especially in elderly patients and, has been recommended by the World Health Organization (WHO), the American Pain Society (APS) [3] and, the American college of Rheumatology (ACR) [4]. Clinical use of combinations of analgesic drugs has increased considerably in the last few years. The purpose of combining two or more drugs with different mechanisms of action is to achieve a synergistic interaction [1], yielding a sufficient analgesic effect with lower doses, and, therefore, reducing the intensity and incidence of untoward effects [5]. At present, many diverse classes of drugs serve as an efficient complement to non-steroidal anti-inflammatory drugs (NSAIDs), acetaminophen or opioids, in the management of pain. And, at the end, the use of these combinations limits the doses of medication that a patient can receive. The evolution of acute pain therapy has prominently included several combinations of analgesics, including acetaminophen and codeine, acetaminophen and oxycodone, ibuprofen and codeine, and several others. The promise of these combinations is well-known, but so, too, are the potential for adverse events [6].

The success of analgesic combination drugs depends on the type of pain that is targeted (acute/chronic, inflammatory, neuropathic, cancer), and evidence suggests that, the combination of Acetaminophen also known as Paracetamol, or N-acetyl-p-aminophenol (APAP) and; Ibuprofen has been found to be efficacious in a variety of acute pain states, including postoperative pain, dysmenorrheal and musculoskeletal pain [6,7]. NSAIDs, such as Ibuprofen, have analgesic, antipyretic and anti-inflammatory actions. They inhibit synthesis of prostaglandins by inhibiting cyclo-oxygenase (COX), present as COX-1 and COX-2. Their analgesic and anti-inflammatory effects are a consequence of COX-2 inhibition [8] and, it's safety/efficacy profile is well characterized, and it has a well-established history of use both as a prescription and as an OTC analgesic [9]. In the post codeine world, [10], the effectiveness of Caffeine (30 mg, 50 mg, 100 mg and 200 mg) as an adjuvant analgesic to Ibuprofen (100 mg or 200 mg) and Acetaminophen has also been evaluated and found to accentuate the potency of Ibuprofen by 140-180% [10-12] in the Ibuprofen-caffeine combination treatment of postoperative pain after removal of third molars. This has led to the production of multi component analgesics preparations involving Aceta-minophen, Ibuprofen and Caffeine by pharmaceutical companies, locally and abroad [13,14].

There are at least 40 different combination analgesic preparations involving Acetaminophen and Ibuprofen in different dosages with or without caffeine all over the world [10], for the management of pain, and this demands the need for proper and efficient analytical methods, since the quality of these combination drug therapies cannot be compromised. At present, there is no existing method of assay for a combination of Acetaminophen, Caffeine, and Ibuprofen in a single dose formulation in the official pharmacopoeias. Some papers have described

the analysis of Paracetamol, Caffeine and Ibuprofen in combination therapy based on titrimetric and UV-Spectroscopic methods [15-17]. Few HPLC with UV, and by capillary electrophoresis with conductivity detection methods are also available for the assay of this combination formulation [18,19]; however, there is no existing method (RP-HPLC-UV) reported for the analysis of Acetaminophen, Caffeine, and Ibuprofen in a fixed dose formulation using cost effective solvents like methanol and phosphate buffer which will ensconced pharmaceutical manufacturing industries, as afar as routine analysis of raw materials and products are concerned.

Hence, the aim of this study was to develop and validate a suitable and, cost effective HPLC-UV method for the simultaneous quantification of Acetaminophen (APAP), Caffeine and Ibuprofen in fixed dose combination drugs using cost effective solvents according to ICH - Q2R1[2005] guidelines. A study of several combinations of different solvents and buffer systems, different pH and suitable internal standards for proper quantitation, led to the described analytical conditions giving sharp and well resolved peaks in a short time. Combination products on the Ghana market containing these analgesics were assayed using the validated method.

2. EXPERIMENTAL

2.1 Instrumentation

The HPLC analyses were carried out on a Knauer Advanced Scientific Instrument (Smartline, Germany), which is equipped with Quaternary Smartline Pump 1000, Smartline degasser, Smartline autosampler 3900, Smartline UV- detector 2500, Smartline Manager 5000, Injection and Switching Valves and, an Eurochrome Software. Chromatographic peaks (UV spectra) were electronically integrated (from 190nm to 400nm for peak identification) and recorded with (Knauer) computing integrator. The column used was a Stationary Vertex Column (Eurospher 100 – $5C_{18}$) - 250 x 4.6mm, with pre-column from Knauer (ASI, Germany) maintained at, > 30°C.

Noise auto detection of the Eurochrome software: There is a check field for noise detection by the Eurochrome software. When activated, Eurochrome® automatically calculates and sets the optimal minimum peak height and width parameters.

2.2 Reagents and Materials

Pharmaceutical grades of Paracetamol (from Tianjin Boafa Pharma. Co. Ltd China), Anhydrous Caffeine (USP Reference Std. - CAT.NO.1085003 USP Rockville. MD LOT K0K210), and Ibuprofen (USP Reference Std. - CAT.NO.1335508 USP Rockville. MD LOT K0J008), were supplied by Letap Pharmaceuticals-(Accra, Ghana), and were certified to have purities of 99.50%, 99.94% and 99.97% respectively. Piroxicam used as an internal Standard (0.04% w/v) in the HLPC procedure was an in-house standard and its purity was certified to be 99.70%. Methanol (HPLC grade- BDH Poole, England), Potassium dihydrogen orthophosphate (HPLC grade BDH Poole, England), orthophosphoric acid and water were doubly distilled from all glass apparatus and used throughout the experiment.

2.2.1 Placebo materials

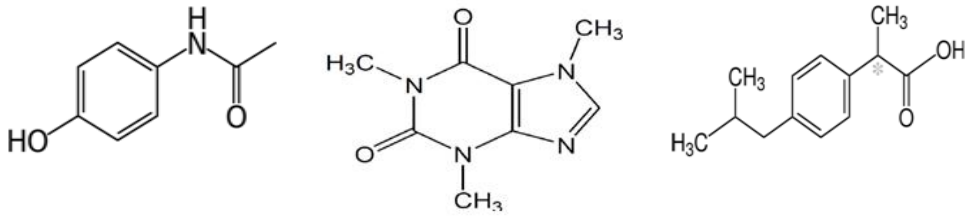
Magnesium Stearate (5 mg - Legend Industries, India), Sodium Lauryl sulphate (4 mg - Aarti Industries Ltd., India), Talc (10 mg- Abhishek Organic Pvt. Ltd., India), and Water (qs).

2.2.2 Commercial samples

Commercial samples except Parabru plus™ - (donated by Letap Pharma. Ltd) were purchased from the local market.

Table 1. Commercial samples analyzed and their existing strength - (API)

Product	Brand/Country	Dosage form	Existing strength of active ingredients		
			Acetaminophen	Caffeine	Ibuprofen
Parabru plus ™	Letap Pharma. Ltd, GH.	Capsules	325 mg	30 mg	200 mg
Combicin	Dev Life Corp., India	Capsules	325 mg	30 mg	200 mg
Maxigesic®	Sigma Laboratories, India	Tablets	500 mg	N/A	150 mg
Sabucap™	Salom Pharma. Ltd, GH.	Capsules	325 mg	30 mg	200 mg
Ibucap	Shalina Lab. PVT. India	Capsules	325 mg	30 mg	200 mg
PocumolExtra™	Poku Pharma Ltd. GH.	Caplets	500 mg	30 mg	40 mg



(A) (C) (I)

Fig 1. Chemical structures of acetaminophen (A), Caffeine (C) and, (RS) Ibuprofen (I)

2.3 Chromatographic Conditions

The mobile Phase was prepared by mixing Methanol and 0.025M Phosphate Buffer- (adjusted to pH 3.2 with orthophosphoric acid), in the ratio of 85:15. The mobile phase was sonicated for 20 mins, filtered, and degassed by passage through a 0.45-µm nylon filter (Millipore, Bedford, MA) under a vacuum. Vertex Column (Europher 100 – $5C_{18}$) 250 x 4.6 mm reversed phase was used and, column effluent was monitored at 225 nm. All determinations were performed at temperature 25°C, pressure < 300 Mpa and wavelength of detection 225nm. The Injection volume was 20 µl with a flow rate of 1.0 ml/min. The sensitivity of the UV -detector was set at 0.1 AUFS (Absorbance Units Full Scale).

2.4 Preparation of Standard Solutions

2.4.1 Internal standard preparation

Weigh accurately 0.04 g of piroxicam powder (reference standard) into a 100 ml volumetric flask; dissolve with 20 ml of the mobile phase, shake to mix, and then make up to volume with the same medium to obtain a concentration of 400 µg/ml.

2.4.2 Acetaminophen-caffeine-ibuprofen stan-dard preparation

The entire validation was carried out on formulation products on the Ghana market, which have existing strengths (API's) of Paracetamol – 325 mg, Caffeine – 30 mg, and Ibuprofen – 200 mg.

Approximately 0.1135 g of Acetaminophen, 0.0105 g of caffeine, and 0.07 g of Ibuprofen reference standards were accurately weighed into three different 100 ml volumetric flasks. The powders were dissolved and made up to volume with the mobile phase – (Stock solution). 4 ml each of the above solutions was pipetted and transferred (combined) into a 25 ml volumetric flask. 5 ml of the internal standard (concentration of 80 µg/ml) was added and the entire solution made up to volume with the mobile phase, to obtain a final concentration of 4540 µg/ml of Acetaminophen, 420 µg/ml of Caffeine, and 2800 µg/ml of Ibuprofen.

2.4.3 Placebo preparation

Weigh an amount of placebo equivalent to 0.1820 g of Parabru plus (TM) capsule content into a 50 ml volumetric flask. Add 10 ml of diluent, sonicate for 5 mins, and top up to the mark with the mobile phase. Filter and pipette 2 ml of the solution into a 25 ml volumetric flask, and top up to the mark.

2.4.4 Preparation of buffer (0.025M Orthophosphoric acid pH 3.2)

Approximately 1.75 g of Potassium dihydrogen orthophosphate was weighed into a 500 ml volumetric flask. 100 ml of distilled water was added and shaken to dissolve. The solution was then topped with water to the 500ml mark, and pH adjusted to 3.2 with orthophosphoric acid.

2.4.5 Diluent

The mobile phase was used as the diluent.

2.5 Method Validation

2.5.1 Instrument precision/ reproducibility

Five (5) repeated injections of a single homogenous standard solution of the combination drug formulation (Acetaminophen-Caffeine-Ibuprofen) with Piroxicam as an internal standard was injected onto the Chromatograph and the Relative Standard Deviation of each compound was calculated.

2.5.2 Specificity

Chromatographic peak precision and spectral purities of Acetaminophen, Caffeine, and Ibuprofen were determined using UV spectra recorded by the smartline UV –detector. In addition, a solution containing a mixture of the tablet excipients was prepared using the sample preparation procedure and injected onto the chromatograph, to ascertain possible interfering peaks.

2.5.3 Linearity and range

Standard solutions containing 4540 µg/ml of Acetaminophen, 420 µg/ml of Caffeine, and 2800 µg/ml of Ibuprofen were prepared in triplicate. Aliquots of these solutions were diluted with the mobile phase to six (6) different concentrations, of Acetaminophen, Caffeine and, Ibuprofen in the assayed range. Solutions of concentration 104 µg/ml, 123.2 µg/ml,181.2 µg/ml, 226 µg/ml, 280 µg/ml and 317 µg/ml of Acetaminophen, 10.2 µg/ml, 11.4 µg/ml, 16.8 µg/ml, 22.0 µg/ml, 27.4 µg/ml and, 31.9 µg/ml of caffeine, and 64 µg/ml, 76 µg/ml, 114 µg/ml, 139 µg/ml, 170 µg/ml, and 210 µg/ml, of Ibuprofen were finally prepared using the mobile phase. Calibration curves for Peak Area Responses against Concentrations (%w/v), were plotted for each compound and the obtained data were subjected to regression analysis.

2.5.4 Intermediate precision

The intra-day precision was evaluated by analyzing six (6) sample solutions (n = 6) at the final concentration of analyses (454 µg/ml of Acetaminophen, 42 µg/ml of Caffeine and 280 µg/ml of Ibuprofen) using Piroxicam as the internal standard (80 µg/ml). Similarly, the interday precision was evaluated in three consecutive days (n = 18) and the Relative standard deviation (R.S.D) was calculated for each component of interest.

2.5.5 Accuracy

By means of standard addition recovery.

Acetaminophen (WHO STD), Caffeine (WHO STD) and, Ibuprofen (WHO STD), were added to a mixture of the tablet excipients and the recovery determined against 75%, 100%, and 125% of the target concentration. Three different concentration levels were prepared, Acetaminophen- (130 µg/ml, 182 µg/ml, and 228 µg/ml), Caffeine- (12.6 µg/ml, 16.8 µg/ml, and 21.0 µg/ml), and Ibuprofen- (84 µg/ml, 112 µg/ml, and 140 µg/ml). At each level samples were prepared in triplicate and the recovery percentage was determined.

Table 2. Chromatographic parameters for acetaminophen, caffeine, piroxicam, and ibuprofen at different mobile phase composition using a vertex column (Europher 100 – 5C_{18}) 250 x 4.6mm reversed phase at 225 nm

Mobile phase composition Methanol: Phosphate buffer (pH 3.2)	Total run time of elution of all compounds/mins	Peak resolution			
		Acetaminophen	Caffeine	Piroxicam (Int. Std)	Ibuprofen
50:50	21	Broad,	Small, co-eluting	Small, overlapping	Broad,Tailing
55:45	19	Broad	Co-eluting	Small	Tailing
60:40	16	Broad	Co-eluting	Small	Tailing
65:35	12	Resolved	Small peak	Small, Resolved	Resolved
70:30	12	Resolved	Slightly resolved	Resolved	Resolved
80:20	11	Well resolved	Resolved	Resolved	Resolved
85:15*	7	Well resolved	Well resolved	Well resolved	Well resolved
90:10	7	Well resolved	Resolved	Well resolved	Resolved
95:05	5	Well resolved	Co-eluting with APAP	Well resolved	Resolved

2.5.6 Robustness

Five sample solutions were prepared and analyzed under the established conditions, and by variation of the following analytical parameters: flow rate- (0.8, 1.0, and 1.2 ml/min) of the mobile phase, proportion of methanol in mobile phase- (83%, 85% and 87%), mobile phase pH- (3.0, 3.2, and 3.4) and column temperature- (20°C, 25°C, and 30°C).The Acetaminophen, Caffeine, and Ibuprofen contents were determined for each condition and the data obtained subjected to statistical analysis (ANOVA).

2.5.7 Detection (LOD), and quantitation (LOQ) limits - (Range)

The entire validation was carried out on a formulation product on the Ghana market by Letap Pharmaceuticals Ltd, Accra, Ghana, which has existing strengths (API's) of Paracetamol – 325 mg, Caffeine – 30 mg, and Ibuprofen – 200 mg.

Combined standards were prepared by sequential dilution and injected onto the chromatograph at decreasing concentrations in the range of 45.4 – 454 µg/ml of Acetaminophen, 4.2 – 42 µg/ml of Caffeine and 28 – 280 µg/ml of Ibuprofen. Since Acetaminophen, which has the highest amount (API) present in the formulation, has a better absorption in the adopted wavelength of detection (225 nm) than Caffeine - which incidentally has the lowest amount (API); the minimum detection limits were set with regards to the detection of Caffeine as the sensitivity of the method.

2.5.8 Efficiency of column (number of theoretical plates)

The number of separate layers (Theoretical plates), with regards to the efficiency of the column (*Vertex Column (Europher100 – 5C_{18}) 250 x 4.6mm* used for the method development and validation was also calculated using the formula $N = 16(t/W)^2$, where N, t and W represent the number of theoretical plates, retention time of the compound, and peak width of the compound's chromatogram respectively.

2.5.9 Tailing factor of chromatographic peaks

The tailing factor for the chromatogram of each component in the formulation product was calculated using the formula:$T_f = W_{0.05}/2f$, where $W_{0.05}$ is the width of the peak at 5% height, and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline [21].

2.6 Analysis of Fixed Dose Combination Tablets/Capsules/Caplets-(Parabru Plus™ Capsule-(Acetaminophen-325 mg, Caffeine- 30 mg, Ibuprofen 200 mg)

Carefully empty and combine the contents of 20 Capsules of the combined formulation drug. Weigh approximately 0.1g of the powder into 50ml volumetric flask. Make up to volume with the mobile phase. Pipette 2ml of the solution into a 25 ml volumetric flask- (final concentration -182 µg/ml, 16.8 µg/ml and 112 µg/ml of Acetaminophen, Caffeine, and Ibuprofen respectively). Add 5ml - (80 µg/ml) of the 0.04% w/v Piroxicam solution (int. std), and make solution up to volume with the mobile phase. The solution was filtered through a 0.45 µm membrane filter [22-29].

3. RESULTS AND DISCUSSION

The chromatographic conditions were initially determined using a *Shim-pack CLC-ODS* (6 mm i.d, x 150) column from Shimadzu (Shimadzu, Kyoto, Japan), and various combinations of methanol/phosphate buffer; and methanol/0.05% trifluoroacetic acid (pH and ionic strength modified with orthophosphoric acid), at different flow rates of 0.5, 1.0, 1.5 and 2.0, in order to optimize column capacity factor for good separation and resolution. A wavelength of maximum absorption measurement was also carried out for peak identification in the range 190 – 400 nm, by injecting 20 µl standard solutions of acetaminophen, caffeine, and Ibuprofen – quantitative amounts as present in the combined formulation product. The strength of each phosphate buffer or trifluoroacetic acid (different pH) was combined with methanol in disparate proportions, starting with a 50:50 combination and gradually increasing and decreasing the aqueous content while monitoring their respective effects on separation and resolution. Some of the above chromatographic conditions could elute the compounds of interest in both the bulk powders and tablet matrix with reasonable retention times, but not the other samples being considered for internal standards -(Aspirin, Salbutamol and Piroxicam). Some had poor resolution and tailing peaks, whilst others had poor resolution and unduly long run times Thus, the Shimadzu C_{18} was substituted with a *Vertex C_{18} column (Eurospher 100 – 250 x*.

Table 3. Instrument precision /reproducibility results

Inj. no.	Acetaminophen peak area	Caffeine peak area	Ibuprofen peak area	Piroxicam peak area	Relative app. acetaminophen	Rel. app. caffeine	Rel. app ibuprofen
1	109.623	7.262	45.936	48.988	2.241	0.148	0.939
2	118.315	8.546	52.8902	54.593	2.167	0.157	0.969
3	119.464	8.606	53.1515	54.866	2.177	0.157	0.969
4	119.116	8.671	53.383	54.983	2.166	0.158	0.971
5	117.309	8.437	52.4982	54.049	2.170	0.156	0.971
ΣX					10.92	0.776	4.819
$\bar{\mu}$					2.184	0.155	0.964
δn					0.028	0.003	0.124
RSD					1.30%	1.9%	1.29%
			SEM	*±0.0125*	*±0.0013*	*±0.0554*	

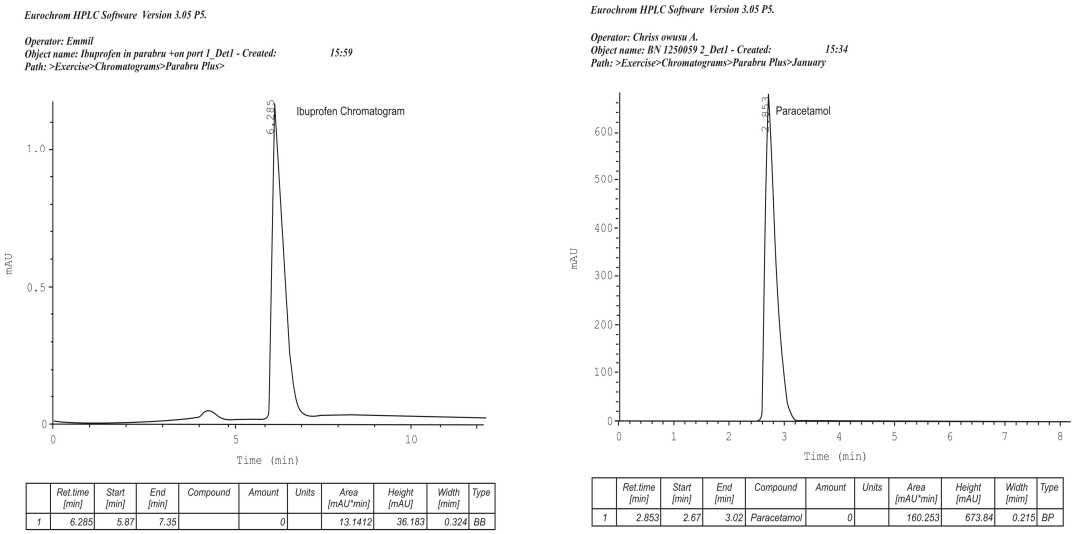
4.6mm). Using this column, and altering the combination ratio, ionic strength and pH of the mobile phase, a reversed phase (HPLC-UV) optimized condition was achieved to provide a specific procedure suitable for rapid quality control, simultaneous quantification of Acetaminophen, Caffeine and, Ibuprofen in combination dosage forms. A Mobile Phase consisting of Methanol and 0.025M Phosphate buffer adjusted to pH 3.2 with othorphosphoric acid in the ratio of 85:15, a Vertex Column (Eurospher 100 – $5C_{18}$), (250 x 4.6 mm) with pre-column, were the optimized conditions with Piroxicam as the internal standard. All determinations were performed at temperature 25°C, pressure < 300 Mpa and wavelength of detection 225 nm. The Injection volume was 20 µl with a flow rate of 1.0ml/min, and the sensitivity was set at 0.1 AUFS (Absorbance Units Full Scale).

3.1 Validation

3.1.1 Instrument precision/reproducibility

Five (5) repeated injections of a single homogenous standard solution of the combination formulation (Table 2) using Piroxicam as the internal standard was made, and the Relative Standard deviations calculated. Relative Standard deviations of 1.30%, 1.90%, and 1.29% were obtained for Acetaminophen, Caffeine and Ibuprofen respectively. The RSD values lower than 2.0% assure the precision of the instrument.

3.1.1.1 Chromatograms



	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [mim]	Type
1	6.285	5.87	7.35		0		13.1412	36.183	0.324	BB

	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [mim]	Type
1	2.853	2.67	3.02	Paracetamol	0		160.253	673.84	0.215	BP

Fig. 2. Chromatograms of acetaminophen and ibuprofen- singly injections onto Chromatograph, using a Mobile Phase of Methanol and 0.025M, Phosphate Buffer adjusted to pH 3.2 with orthophosphoric acid in the ratio of 85:15. Vertex Column (Eurospher 100 – 5C18) 250 x 4.6 mm reversed phase and the column effluent was monitored at 225 nm

Table 4. Statistical results for linearity of investigated compounds Y = (B + *Sb*)*X + (A + *Sa*)

Parameters	Acetaminophen	Caffeine	Ibuprofen
Slope'B' ± *Sb*	11602.3 ± 368.0921	8528.937 ± 406.1064	6361.683 ± 175.8732
Intercept 'A' ± *Sa*	-28.2418 ± 8.073883	-1.54139 ± 0.872452	6.245365 ± 2.43509
R^2	0.9960	0.991	0.9970
$S_{y/x}$	6.994799	0.793299	2.199477
Fisher's *F*	993.5168	441.0727	1308.409
v	4	4	4
Conc. Range (µg/ml)	104.0 – 317.0	10.2 – 32.0	60.0 – 210.0

Sb = Standard error of the slope, Sa = Standard error of the Intercept; v = degrees of freedom, $S_{y/x}$ = Standard error of the regression, R^2 = Correlation Coefficient

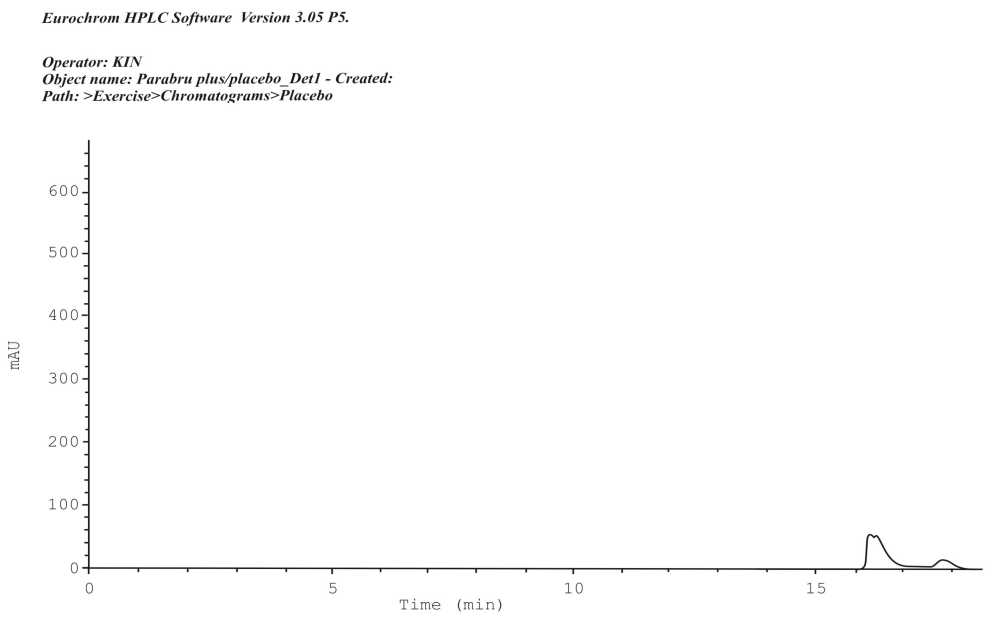
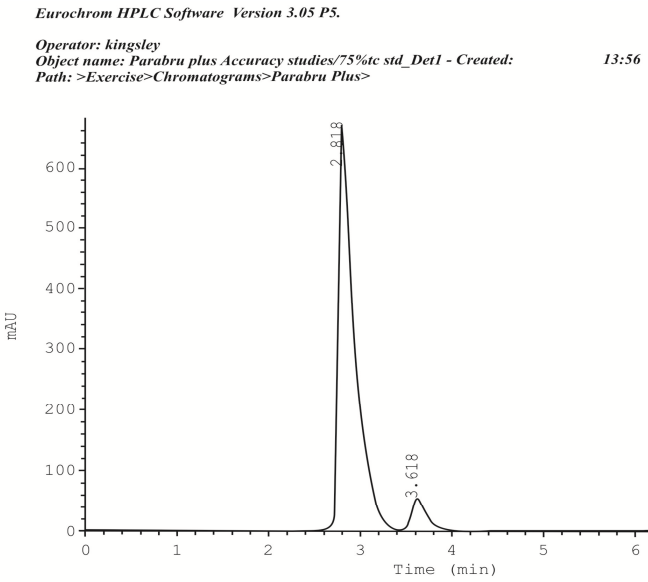
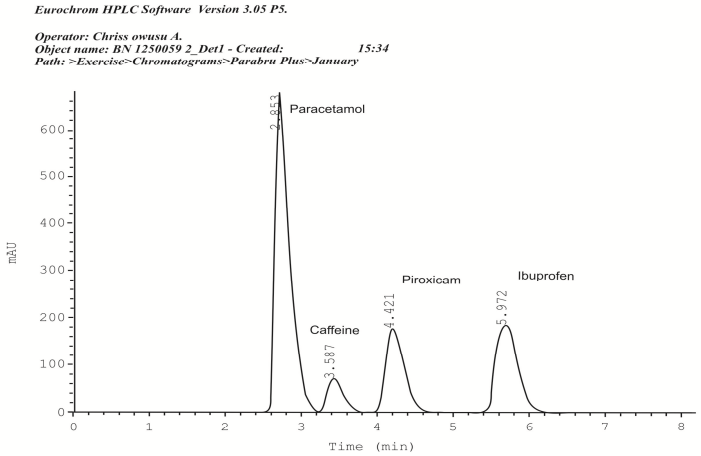


Fig. 3. Chromatogram of 40 ul placebo injection onto chromatograph, using a mobile phase of Methanol and 0.025M, phosphate buffer adjusted to pH 3.2 with orthophosphoric acid in the ratio of 85:15. Vertex Column (Eurospher 100 – 5C_{18}) 250 x 4.6 mm reversed phase and the column effluent was monitored at 225 nm



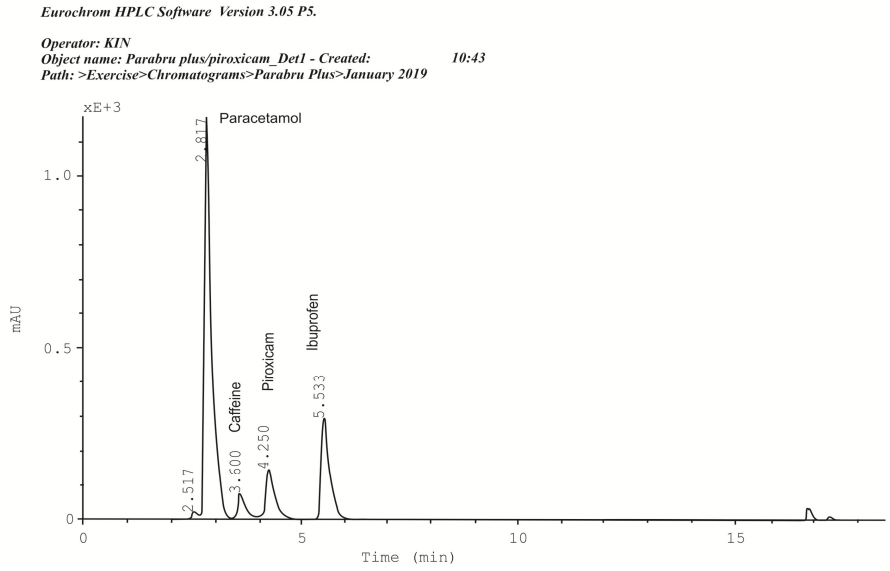
	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [mim]	Type
1	2.818	2.68	4.07	Paracetamol	0		139.378	677.725	0.172	BB
2	3.618	3.47	4.05	Caffeine	0		11.2826	52.4333	0.194	SS
3	6.603	6.19	7.75	Ibuprofen	0		61.5834	211.542	0.260	BB

Fig. 4. Retention times of paracetamol, caffeine, and ibuprofen injection studied, using a mobile phase of methanol and 0.025M, phosphate buffer adjusted to 3.2 with orthophosphoric acid in the ratio of 85:15. Vertex column (Eurospher 100-5 C_{18}) 250 × 4.6 mm, and the column effluents were monitored at 225 nm



	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [mim]	Type
1	2.853	2.67	3.21	Paracetamol	0		160.253	673.84	0.215	BP
2	3.587	3.39	4.00	Caffeine	0		16.9544	67.9096	0.239	SS
3	4.421	4.02	5.52	Piroxicam	0		54.5444	177.278	0.282	PB
4	5.972	5.59	7.44	Ibuprofen	0		67.8005	185.62	0.347	BB

Fig. 5. Chromatogram of paracetamol, caffeine, piroxicam, and ibuprofen injection, using a mobile phase of methanol and 0.025M, Phosphate Buffer adjusted to pH 3.2 with orthophosphoric acid in the ratio of 85:15. Vertex column (Eurospher 100 – 5C_{18}) 250 x 4.6 mm. Column effluent was monitored at 225 nm



	Ret.time [min]	Start [min]	End [min]	Compound	Amount	Units	Area [mAU*min]	Height [mAU]	Width [mim]	Type
1	2.517	2.30	2.70	Solvent	0		3.14469	17.9683	0.112	BP
2	2.817	2.70	3.41	Paracetamol	0		210.075	1168.68	0.144	PB
3	3.600	3.47	3.93	Caffeine	0		12.1741	67.7642	0.151	SV
4	4.250	3.93	5.05	Piroxicam	0		32.4252	140.682	0.183	VS
5	5.533	5.25	6.25	Ibuprofen	0		64.7888	289.368	0.196	SS
6	16.873	16.56	17.25	Impurity	0		3.245	17.9781	0.164	BP

Fig. 6. Chromatogram of acetaminophen, caffeine, piroxicam and ibuprofen in an assayed product using mobile phase of methanol and 0.025M, phosphate buffer adjusted to pH 3.2 with orthophosphoric acid in the ratio of 85:15. Vertex column (Eurospher 100 – 5C_{18}) 250 x 4.6 mm. The column effluents were monitored at 225nm

3.1.2 Linearity

A linear correlation was found between the peak area responses and the concentrations of Acetaminophen, Caffeine and Ibuprofen in the assayed range. The regression coefficient (r) obtained are 0.9960, 0.9910, 9971 for Acetaminophen, Caffeine and Ibuprofen respectively. These regression coefficients (r) were higher than 0.99, which confirmed the linearity of the method. The residual plots were also normally distributed around the zero mark. The regression analysis data and calibrations graphs are presented below.

3.1.2.1 Residual plots (Calculated from data generated for Linearity)

The analysis of residuals for Acetaminophen, Caffeine and, Ibuprofen in the combination formulation with the tablet excipients showed that the values are scattered randomly around zero, indicating the linearity of the method. See plots below.

3.1.3 Specificity

Peak precision and purity around 99% to 100% were obtained for Acetaminophen, Caffeine and Ibuprofen in the chromatogram of sample solutions (standards) and mixture of standards with tablet excipients. The chromatograms obtained with the mixture showed no interfering peaks in the same retention time for the Acetaminophen, Caffeine and Ibuprofen, indicating that, other compounds such as the excipients do not co-elute with the main peaks.(Fig. 3.), (Fig.4.), and (Fig. 5).

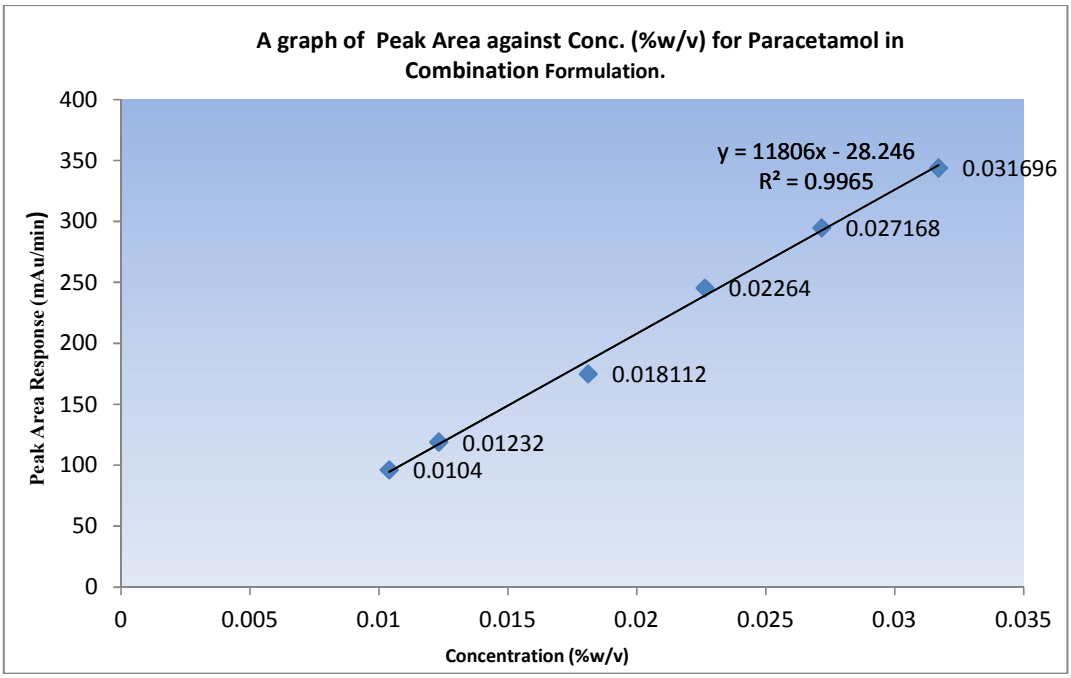
3.1.4 Repeatability

Six independent weights of Acetaminophen, Caffeine and Ibuprofen (std) at 100% concentration using piroxicam as the internal standards were injected. The RSD on statistical evaluation for the six injections was < 2.0% for all the three components of interest. Relative standard deviations of 1.18%, 1.30%, and 1.06% were obtained for Acetaminophen, caffeine and Ibuprofen respectively.

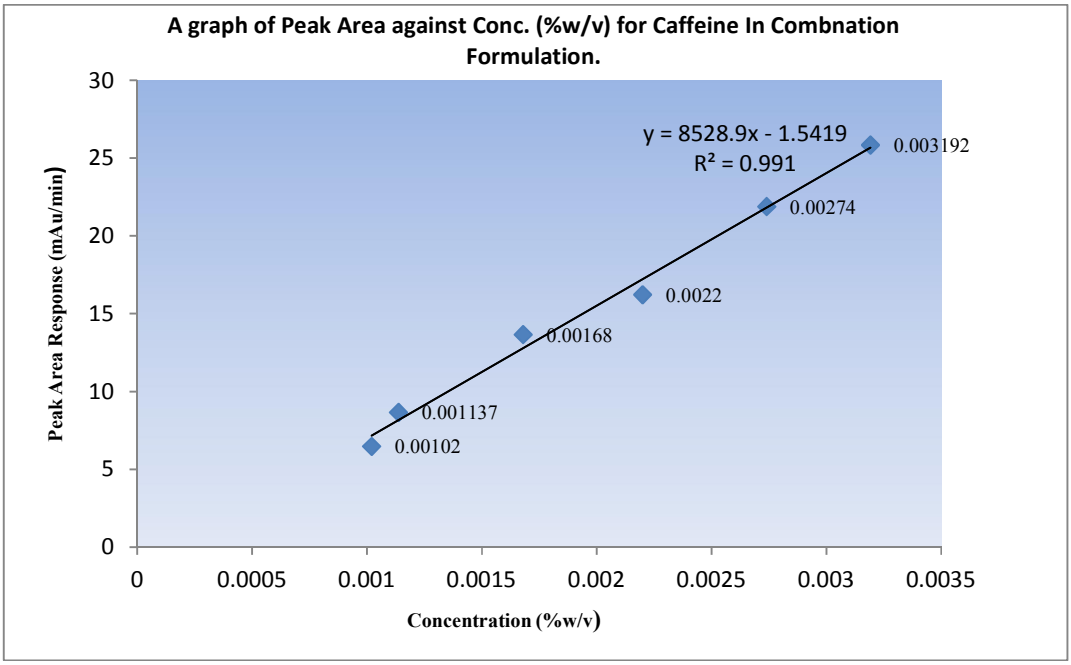
3.1.5 Accuracy

By means of standard addition recovery.

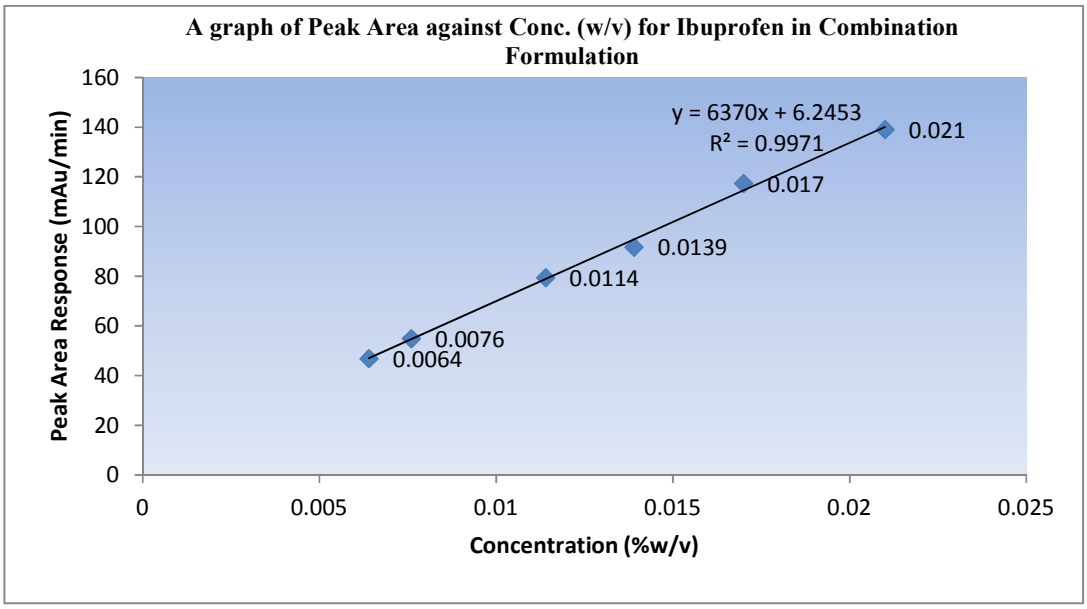
Acetaminophen (WHO STD), Caffeine (WHO STD), and Ibuprofen (WHO STD) were added to a mixture of the tablet excipients and determined against 75%, 100% and 125% of the target concentration. The mean recoveries of three sets of injections (n = 9) for Acetaminophen, Caffeine and Ibuprofen were 99.43%, 99.84% and 99.28% respectively, indicating the accuracy of the method.



7A.



7B.



7C.

Fig. 7. A, B, and C: Calibration (Linearity) graphs for acetaminophen, caffeine, and ibuprofen in fixed dose combination formulation with tablet excipients, respectively

Table 5. Results for LOD and LOQ

Parameters	Acetaminophen (µg/ml)	Caffeine (µg/ml)	Ibuprofen (µg/ml)
LOD	45.4	4.2	28.0
LOQ	454.0	42.0	280.0

Table 6. i, ii, and iii

Table for residual plots– (a combination formulation of Paracetamol, Caffeine and, Ibuprofen)

i. Acetaminophen, from the equation of the straight line y = 11806x – 28.24

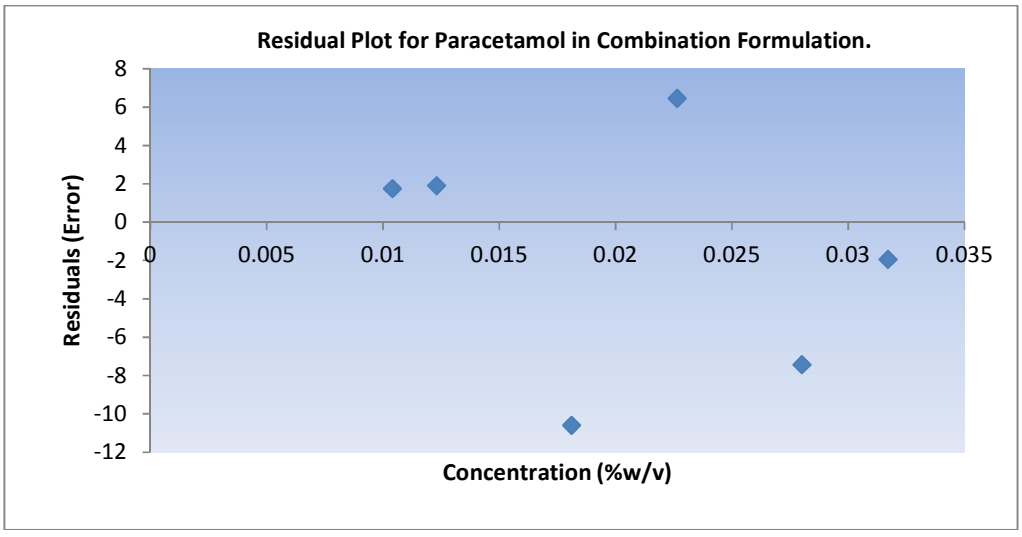
Concentration (x-%w/v)	Y- observed value (Yo)	Y- predicted value (Yp)	Residuals (Error) (Yo – Yp)
0.0104	96.2932	94.5364	1.7568
0.0123	119.116	117.2039	1.9121
0.0181	174.990	185.5843	-10.5940
0.0226	245.507	239.0418	6.4652
0.0280	294.889	302.3220	-7.4330
0.0317	344.017	345.9570	-1.9400

ii. Caffeine, from the equation, y = 8528x – 1.541

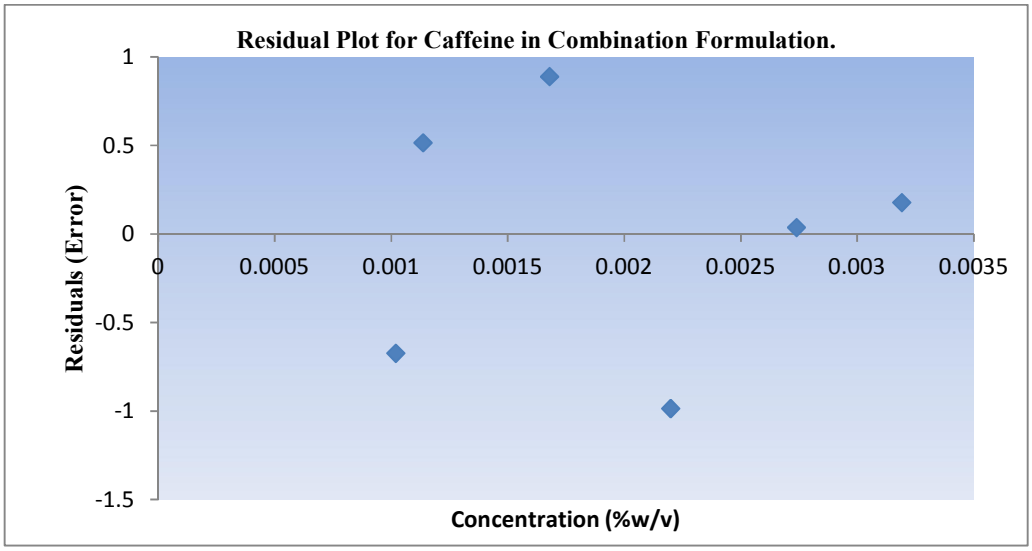
Concentration (x-%w/v)	Y- Observed value (Yo)	Y- Predicted value (Yp)	Residual (Error) (Yo – Yp)
0.00102	6.4850	7.15756	-0.67256
0.001137	8.67120	8.155336	0.515864
0.00168	13.6757	12.78604	0.889660
0.0022	16.2361	17.22060	-0.98450
0.00274	21.9450	21.82572	0.04730
0.003192	25.859	25.680376	0.178624

iii. Ibuprofen, from the equation, y = 6370x + 6.245

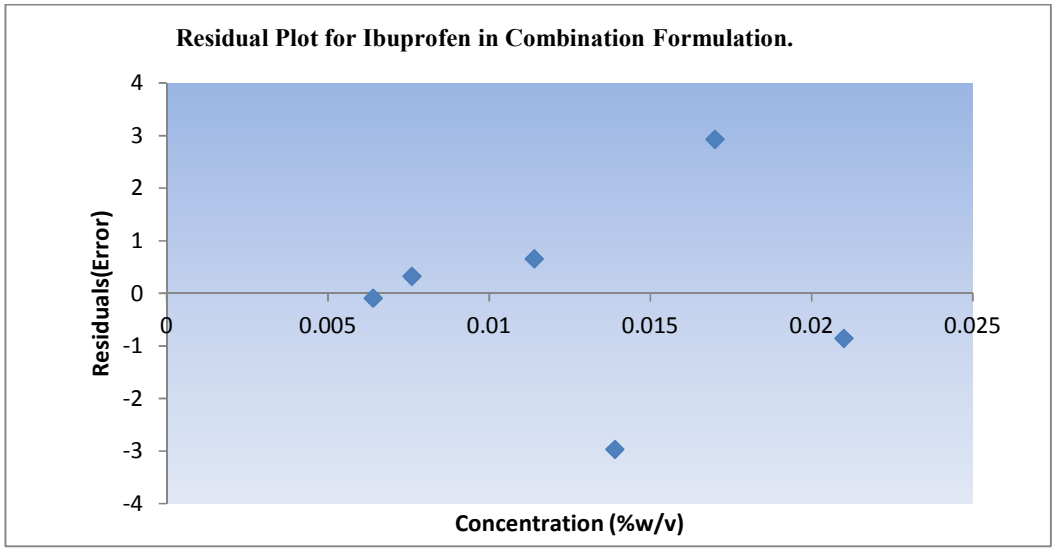
Concentration (x - %w/v)	Y- observed value (Yo)	Y- Predicted value (Yp)	Residual (Error) (Yo – Yp)
0.0064	46.9228	47.0130	-0.0902
0.00758	54.9827	54.6570	0.32570
0.01134	79.5202	78.8630	0.65720
0.0139	91.8231	94.7880	-2.96490
0.017	117.4660	114.5350	2.9310
0.021	139.16035	140.0150	-0.85460



8.A



8.B



8.C

Fig. 8.- A, B and C: Residual plots for acetaminophen, caffeine, and ibuprofen in combination formulation respectively

3.1.6 Intermediate precision

In the intra-day precision analysis (n = 6), mean contents of Acetaminophen, Caffeine, and Ibuprofen were 453.8-(R.S.D. = 1.17%), 42.40-(R.S.D. = 1.54%) and, 279.30-(R.S.D. = 0.98%), respectively. The inter-day precision (n = 18), also had mean contents of 451.8-(R.S.D. = 1.13%), 43.10-(R.S.D. = 0.94%), and 280.78- (R.S.D. = 1.28%) for Acetaminophen, Caffeine, and Ibuprofen respectively. Relative Standard Deviation values were less than 2%, indicating the precision of the method.

3.1.7 Robustness

The method was robust with a 2% variation in parameters of the analytical conditions established for the method. The statistical analysis showed no significant difference between results obtained. Hence, the method

Table 7. Results of repeatability studies

No	Acetaminophen STD			Caffeine STD			Ibuprofen STD		
	W(g)	Conc. % w/v	Response (X)	W(g)	Conc.% w/v	Response (X)	W(g)	Conc.% w/v	Response (X)
1	0.1130	0.009040	95.3853	0.0105	0.00168	13.4276	0.0701	0.00560	43.1535
2	0.1132	0.009056	98.4127	0.0104	0.00166	13.1086	0.0705	0.00564	42.6221
3	0.1134	0.009072	97.3086	0.0106	0.00170	12.8745	0.0703	0.00562	43.9904
4	0.1131	0.009048	97.8852	0.0104	0.00168	13.0613	0.0704	0.00563	43.7303
5	0.1134	0.009072	96.0444	0.0103	0.00165	13.2116	0.0704	0.00563	43.6869
6	0.1135	0.009080	95.6824	0.0105	0.00168	13.0898	0.0703	0.00562	42.7641
ΣX			580.7186			78.7734			260.9473
$\overline{\mu}$			96.78643			13.1289			43.4912
δn			1.144			0.16698			0.4634
RSD			1.18%			1.30%			1.06%
SEM			±2.802			±0.409			±0.019

ΣX = Sum of Peak areas; δn = Standard Deviation, RSD= Relative standard Deviation

$\overline{\mu}$ = Mean of Peak areas SEM = Standard Error of the Mean

Table 8. A, B, and C: Accuracy studies for method Validation (Using a formulation of Paracetamol, Caffeine, and Ibuprofen combination product– Parabru plus™ capsule)

A. Acetaminophen

Conc. In Parabru plus	Amount added	Amount recovered	% Recovery
1.75% - 130 µg/ml	19.2 µg/ml	18.85 µg/ml	98.19%
2.	19.2 µg/ml	19.40 µg/ml	101.04%
3.	19.2 µg/ml	18.73 µg/ml	97.55%
1. 100% - 182 µg/ml	19.2 µg/ml	19.45 µg/ml	101.33%
2.	19.2 µg/ml	19.30 µg/ml	100.52%
3.	19.2 µg/ml	19.12 µg/ml	99.58%
1. 125% - 228 µg/ml	19.2 µg/ml	18.95 µg/ml	98.68%
2.	19.2 µg/ml	18.78 µg/ml	97.80%
3.	19.2 µg/ml	19.24 µg/ml	100.21%
Average % Recovery across all levels ± SEM			99.43% ± 0.475

B. Caffeine

Conc.In parabru plus	Amount added	Amount recovered	% Recovery
1.75% - 12.6 µg/ml	30.0 µg/ml	31.05 µg/ml	103.50%
2.	30.0 µg/ml	29.98 µg/ml	99.93%
3.	30.0 µg/ml	30.46 µg/ml	101.53%
1. 100% - 16.8 µg/ml	30.0 µg/ml	30.23 µg/ml	100.77%
2.	30.0 µg/ml	29.82 µg/ml	99.40%
3.	30.0 µg/ml	29.45µg/ml	98.16%
1. 125% - 21.0 µg/ml	30.0 µg/ml	29.58µg/ml	98.60%
2.	30.0 µg/ml	30.04 µg/ml	100.13%
3.	30.0 µg/ml	28.96 µg/ml	96.53%
Average % Recovery across all Levels ± SEM			99.84 %± 0.674

C. Ibuprofen

Conc. In parabru plus	Amount added	Amount recovered	% Recovery
1.75% - 84 µg/ml	24.02 µg/ml	24.12 µg/ml	100.42%
2.	24.02 µg/ml	23.56 µg/ml	98.08%
3.	24.02 µg/ml	23.64 µg/ml	98.84%
1. 100 %- 112 µg/ml	24.02 µg/ml	23.70 µg/ml	98.67%
2.	24.02 µg/ml	24.14 µg/ml	100.50%
3.	24.02 µg/ml	23.98 µg/ml	99.83%
1. 125 % - 140 µg/ml	24.02 µg/ml	23.72 µg/ml	98.75%
2.	24.02 µg/ml	23.26 µg/ml	101.0%
3.	24.02 µg/ml	26.40 µg/ml	97.42%
Average % Recovery across all Levels± SEM			99.28% ± 0.405

showed to be robust for flow rate between 0.8 to 1.2 ml/min, Mobile phase proportion for methanol 83 to 87 ml, pH 3.0 to 3.4 and column temperature 25°C to 30°C.

3.1.8 Detection and quantitation limits

The objective of the method is the simultaneous quantification of Acetaminophen, Caffeine, and Ibuprofen in the fixed dose combination tablet/capsule. Since Acetaminophen which, has the highest amount (API) present in the formulation, has a better absorption in the adopted wavelength of detection than caffeine-which incidentally has the lowest amount (API), the minimum detection limits were set with regards to the detection of caffeine as the sensitivity of the method based on the noise-to-signal ratio. Hence, the detection limits of Acetaminophen, Caffeine, and Ibuprofen were 45.4 µg/ml, 4.2 µg/ml, and 28 µg/ml respectively. The quantitation limits were also 454 µg/ml, 42

Table 9. Content of fixed dose, combination from different pharmaceutical manufacturing companies

Product dosage form	Brand/Country	Content (%) ± standard deviation N		
		Paracetamol	Caffeine	Ibuprofen
Combicin Capsules	Dev Life Corporation, India	102.70±0.46	98.96±1.23	100.34±0.65
Ibucap Capsules	Shalina Laboratories PVT, India	102.64±1.08	103.58±0.97	97.87±0.92
Parabru plus™ Capsules	Letap Pharm. Ltd , Ghana	101.19±1.02	97.35±0.72	103.88±0.79
Maxigesic® Tablets	Sigma Laboratories, India	99.54±0.78	N/A	98.75±0.82
Sabucap™ Capsules	Salom Pharmacy Ltd, hana	99.70 ± 0.54	101.60 ± 0.46	99.52 ± 0.16
Pocumol Extra™ Caplets	Poku Pharma Ltd, hana	101.20 ± 0.45	98.40 ± 0.33	99.10 ± 0.62

µg/ml, and 280 µg/ml for the Acetaminophen, Caffeine, and Ibuprofen respectively (Table 4).

3.1.9 Efficiency of the column

The theoretical plates (N) of the column, with respect to Acetaminophen, Caffeine, and Ibuprofen (Fig. 3) in the fixed dose formulation were 4295, 5565 and, 10319 respectively. All (N) values were above 2000, indicating that, the chromatographic column contains a large number of separate layers, hence efficient.

3.1.10 Tailing factor of chromatographic peaks

The tailing factors for the chromatographic peaks of Acetaminophen, Caffeine, and Ibuprofen were 1.0076, 1.0842, and 1.0091 respectively. All T_f values were approximately 1.0, indicating acceptable peak symmetry (Fig. 5) for the chromatograms.

3.1.11 Stability of solutions

Prepared samples (analytes) and Mobile phase are stable over a 72 hour period if stored at room temperature.

3.2 Analysis of Fixed Dose Combination Tablets

Fixeddose formulation of tablets/ capsules/ caplets from six different manufacturing companies, both locally and abroad on the local market (Paracetamol-325 mg, Caffeine-30 mg, and, Ibuprofen-200 mg) was analyzed using the validated method with piroxicam as the internal standard. The content of Acetaminophen, caffeine and Ibuprofen in the tablets/Caplets/ capsules varied from 97.0%- 104.0%.

4. CONCLUSION

The developed method showed to be economical and, a suitable technique to quantify analgesic fixed dose combination therapy and; might be employed for quality control analysis. The method is specific for the detection and estimation of the active ingredients. The method is linear in the specified range. It is also precise. The accuracy of the method is also established, hence this method stands validated with respect to ICH Q2 (R1) 2005 guidelines, and that it is fit for use in the analysis of combination formulations of Acetaminophen, Caffeine and, Ibuprofen and other matrices. The Aceta-minophen, Caffeine and Ibuprofen formulations assayed with the validated method evinced quality and the right amount of active ingredient per the labelled claim.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This work presents no ethical issues that specifically need addressing.

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COMPETING INTERESTS

Authors have declared that no competing interests exist. The products used for this

research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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