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A new gradient HPLC stability indicating method for related substances of Paracetamol, Caffeine and Codeine in effervescent tablet in a single run

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ABSTRACT: Background: Developing a single analytical method for the estimation of individual drugs from a multidrug composition is a very challenging task. There is no related substance analytical method for effervescent triple component formulation specified officially in any of the pharmacopoeias. **Aim:** The present study aimed to develop a simple, rapid, precise, and reliable reverse phase HPLC method for the separation and estimation of three active moieties paracetamol, codeine phosphate, and caffeine for an effervescent dosage form. **Method:** The estimation was carried out using column; Inertsil ODS-3V (250 × 4.6 mm, 5 µm), mobile phase-A consisting of a buffer consisting of 10 mM octane, sodium salt, and 10 mM Potassium phosphate buffer solution at pH (adjusted 2.5 with phosphoric acid), mobile phase-B that is methanol: Acetonitrile: Water (45:45:10) with gradient flow rate and ultraviolet detection at 245 nm with an acquisition time of 80 min. All the three active moieties were properly resolved to having a retention time of 11, 19, and 49 min for paracetamol, caffeine, and codeine respectively. **Result**: The method was validated in terms of specificity, precision, linearity, LOQ and LOD, accuracy, ruggedness, and robustness. **Discussion:** The developed method was validated according to ICH guidelines and values of accuracy, precision, and other statistical analysis were found to be in good accordance with the specified acceptance criteria. **Conclusion:** The proposed method was successfully applied to the triple combination effervescent dosage form for routine analysis.

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INTRODUCTION:

Drug combinations are single preparations containing two or more active pharmaceutical ingredients (APIs) for concurrent administration as a fixed-dose drug [1]. Most multicomponent drug formulations usually contain two or more active ingredients which are responsible for a combined therapeutic activity of the drug. This concept is beneficial when the selective agents have different mechanisms of action that provide additive or

synergistic efficacy [2]. There is increased production of multicomponent drug formulation due to increased efficacy, increased resistance of microorganisms to single component formulations, and dependency and/or tolerance, and this has further led to increased drug counterfeiting and adulteration [3,4].

However, monographs in the most official pharmacopoeia are for single component drugs, hence pharmaceutical manufacturing companies in the analysis of multi-component drug formulations use methods that involve multiple and repeated extractions to extract each active component before their quantification using spectrophotometry or titrimetry. Such methods are thus laborious and cumbersome [5,6]. This has led to researchers developing various methods to help facilitate easy and quick analysis of multi-component drugs. With HPLC being a method of choice, many researchers have worked on developing various RP-HPLC methods for the simultaneous estimation of various active components in multi-component drugs [7,8].

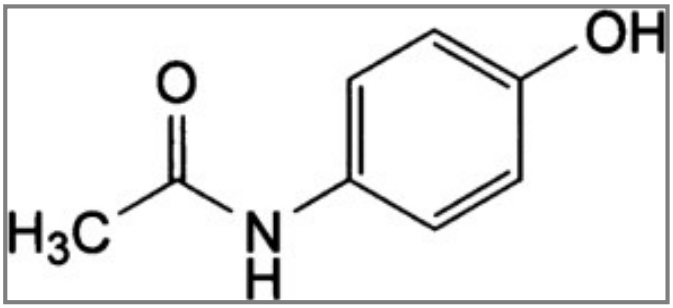

Fig 1. Structure of Paracetamol.

Paracetamol (acetaminophen), N-(4-Hydroxyphenyl)-acetamide (Fig 1) is a widely used analgesic and antipyretic agent for the relief of fever, headaches, minor pains, etc. It is a major ingredient in numerous cold and flu remedies. In combination with non-steroidal anti-inflammatory drugs and opioid analgesics [9,10]. Paracetamol is used also in the management of severe pain (such as post-operative pain). Paracetamol alone or in combination with other drugs is reported to be estimated by titrimetry, spectrophotometric method, HPLC, TLC, HPTLC, UHPLC, LC-MS, FT-IR, amperometric determination, and fluorimetry [11,12].

Codeine phosphate (7,8-Didehydro-4,5a-epoxy-3-methoxy-17- methylmorphinan-6a-ol) phosphate is predominant alkaloid in opium. It is considered a pro-drug, metabolized to active compounds of morphine and codeine-6-glucoronide. Codeine (Fig 2) is the traditional choice for the treatment of moderate opioid-sensitive pains [13,14]. Codeine phosphate in combination with other compounds has been determined in different pharmaceutical preparations by GLC, TLC, UV, and HPLC.

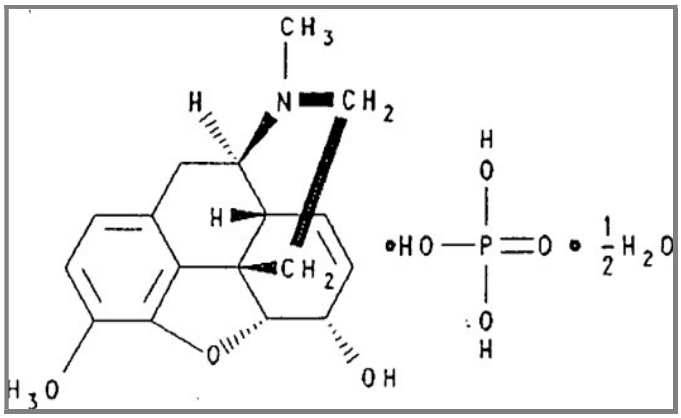

Fig 2. Structure of Codeine phosphate.

Combinations of Codeine with Paracetamol produce a significant increase in analgesia compared with Paracetamol alone. These pharmaceutical formulations accounted for 20 % of total non-opiate analgesics during the last decade. Their quality control is thus of paramount importance, especially the determination of Paracetamol in pharmaceuticals has been critically reviewed since its overdose can cause fulminating hepatic necrosis and other toxic effects [15,16].

Caffeine is a central nervous system (CNS) stimulant of the methylxanthine class. It is the world's most widely consumed psychoactive drug. Unlike many other psychoactive substances, it is legal and unregulated in nearly all parts of the world. There are several known mechanisms of action to explain the effects of caffeine [17,18]. The most prominent is that it reversibly blocks the action of adenosine on its receptor and consequently prevents the onset of drowsiness induced by adenosine. Caffeine (Fig 3) also stimulates certain portions of the autonomic nervous system [19,20].

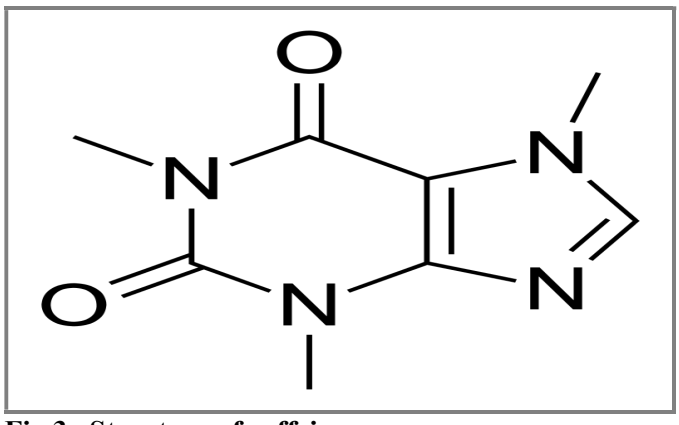

Fig 3. Structure of caffeine.

Caffeine is a bitter, white crystalline purine, a methylxanthine alkaloid, and is chemically related to the adenine and guanine bases of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It is found in the seeds, nuts, or leaves of a number of plants native to Africa, East Asia, and South America, and helps to protect them against predator insects and to prevent germination of nearby seeds [20,21]. The most well-known source of caffeine is the coffee bean, a misnomer for the seed of Coffee plants. Beverages containing caffeine are ingested to relieve or prevent drowsiness and to improve performance [22]. To make these drinks, caffeine is extracted by steeping the plant product in water, a process called infusion. Caffeine-containing drinks, such as coffee, tea, and cola, are very popular; as of 2014, 85 % of American adults consumed some form of caffeine daily, consuming 164 mg on average [23,24].

The main objective of this work is to develop and validate a new, simple, accurate, linear, precise, specific, robust, sensitive, and cost-effective RP-HPLC method for simultaneous estimation of paracetamol, codeine phosphate, and caffeine in multi-component effervescent tablet dosage form.

MATERIALS AND METHODS:

Chemicals and reagents:

Paracetamol, Caffeine, and Codeine phosphate working standards were used available in Oman Pharmaceutical Products L.L.C. Tablet formulations containing Codeine phosphate hemihydrate 8 mg, Caffeine 30 mg, and Paracetamol 500 mg were taken from the Oman Pharmaceutical Products L.L.C. HPLC grade Methanol and Acetonitrile was procured from Merck Ltd. All other chemical reagents were of analytical grade.

Table 1. Gradient program.

Time (min)	Flow rate	Phase-A (%)	Phase-B (%)
0	1.0	90	10
40	1.0	65	35
50	1.0	75	25
55	1.0	75	25
60	1.0	60	40
65	1.0	40	60
70	1.0	90	10
80	1.0	90	10

Preparation of Mobile phase A:

A buffer solution containing 10 mM octane sodium salt, and 10 mM potassium phosphate was prepared. About 2.16 g octane sodium salt and 1.36 g potassium phosphate were weighed and transferred into a beaker containing 1000 ml of Milli-Q grade water and mixed. The pH was adjusted to 2.5 with orthophosphoric acid. The solution was filtered through a 0.45 µ nylon membrane filter and then sonicated for 15 min. The prepared mobile phase was considered mobile phase A. The mixed form of methanol, acetonitrile, and water in the ratio of 45: 45: 10, was considered mobile phase B (Table 1).

Diluent:

About 1.6 g of butane sulfonic acid sodium salt was weighed and mixed with the mixture of 850 ml of water, 150 ml of methanol, and 4 ml of orthophosphoric acid.

Paracetamol, Caffeine, and Codeine standard solution preparation:

About 100, 6, and 45 mg of paracetamol, caffeine, and codeine working standard were weighed accurately and taken in a 100 ml volumetric flask. To the flask, a sufficient amount of methanol was added and then sonicated to dissolve. The solution was diluted up to the volume mark with methanol. About 2 ml of the above solution was transferred into a 100 ml volumetric flask and diluted up to the volume mark with diluent.

Placebo solution preparation:

About 500 mg of paracetamol was weighed as a placebo and taken in a 50 ml volumetric flask. To the flask, 2 ml of diluent was added and waited till effervescence ceased. Then, 10 ml each of diluent and methanol were added and sonicated for about 10 min to dissolve and then diluted up to the mark with diluent. The resulting solution was filtered through a 0.45 µm Nylon filter after discarding the first 5 ml filtrate.

Sample solution preparation:

About 20 tablets were crushed and weighed. About 3.25 g of sample (equivalent to about 500 mg of Paracetamol) was taken in a 50 ml volumetric flask, into which 2 ml of diluent was added and waited till effervescence ceased. Then, 10 ml of each diluent and methanol were added, sonicated for about 10 min to dissolve, and the solution was diluted up to the mark with diluent. The resulting solution was filtered through a 0.45 µm Nylon filter after discarding the first 5 ml filtrate.

Evaluation:

The chromatographic conditions are given in Table 2. The samples were tested for system suitability, specificity, precision, ruggedness, Linearity, range, LOQ, LOD, accuracy, and Robustness [25-29].

Table 2a. Chromatographic conditions.

Column:	Inertsil: ODS-3V, 250 × 4.6 mm, 5µm (Part No: 5020-01802) (Mfg. By G L Sciences)
Pre-column:	Ghost-Buster,4.6 × 50mm (Cat No: 06100-31000) (Mfg. By Welch Materials)
Flow rate:	1.0 ml/min
Injection volume:	10 µL
Wavelength:	245 nm
Column temp:	35 °C
Sampler temp:	5 °C

RESULTS AND DISCUSSION:

The developed method for related substances determination of Paracetamol, Caffeine, and Codeine was validated by using the following parameters.

Table 2b. System suitability – Paracetamol.

Inj #	Area	Tailing factor	Theoretical Plates
1	835562	1.01	22794
2	830728	1.01	22795
3	821443	1.01	22761
4	816485	1.01	22593
5	815122	1.01	22816
6	814835	1.01	22584
Mean	822363	1.01	22724
SD	8816.881	-	-
%RSD	1.1	-	-

Table 2b. System suitability – Caffeine.

Inj #	Area	Tailing factor	Theoretical Plates
1	9499	0.97	55392
2	9794	0.99	53991
3	9366	0.98	55034
4	9678	1.00	53303
5	9847	0.96	52721
6	9803	0.99	53042
Mean	9665	0.98	53914
SD	192.839	-	-
%RSD	2.0	-	-

System suitability:

System suitability followed the procedure described in the methodology and establish the system suitability before starting the analysis. The standard solution is as mentioned in Table 2b, 2c, and 2d.

Table 2c. System suitability – Codeine.

Inj #	Area	Tailing factor	Theoretical Plates
1	53040	1.04	116377
2	53530	1.05	114843
3	52539	1.03	117223
4	52474	1.03	116649
5	53213	1.03	115473
6	52693	1.03	116547
Mean	52915	1.04	116185
SD	416.604	-	-
% RSD	0.8	-	-

Specificity:

There were no interfering peaks in the retention times of the Paracetamol, Caffeine, and Codeine in the presence of excipients. Further, to demonstrate the specificity of the method, the sample had been subjected to acid, base, oxidation, thermal and photolytic degradation. This was evaluated by comparing the purity angle with the purity threshold. The result is given in Fig 20 to 24 for the chromatograms and Tables 2a and 2b for the peak purity data. The force degradation analysis data is given in Table 11a to 11c.

Precision (Unspike Sample):

Precision was determined by preparing the standard and sample as per the methodology. The sample was prepared in six replicates and injected into the chromatograph. Calculate the percentage specified and unspecified impurity for each preparation. Deduce % RSD for percentage specified and percentage unspecified impurity. The data obtained for the six sample preparations have been presented in Table 3 and Fig 12 to 15 for the chromatograms.

Precision (Spike Sample):

Spike Precision was determined by preparing the standard and sample as per the methodology. Prepare sample in six replicates as per the proposed method by spiking 4-aminophenol, 4-Chloroacetanilide, Caffeine Impurity-E, Codeine Impurity-I, Codeine Impurity-J at the specification level (0.01, 0.1 and 1.5 % with respect to sample concentration) and inject into the chromatograph.

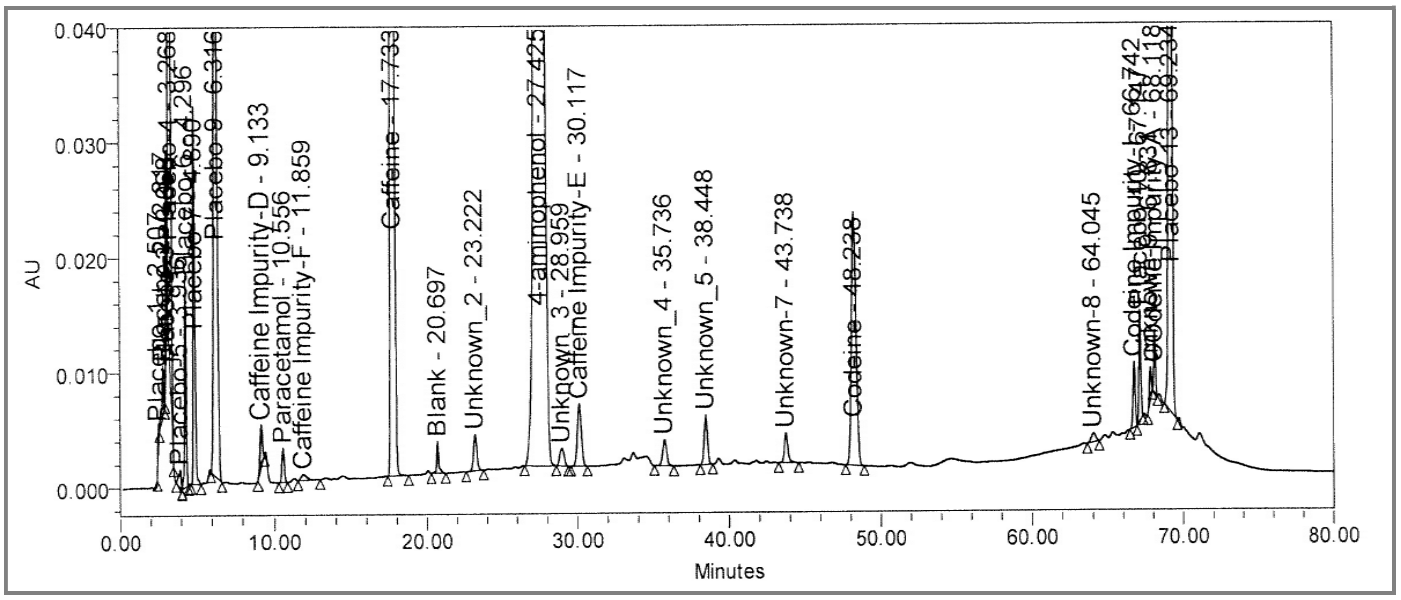

Fig 20. Reference chromatogram of acid degradation.

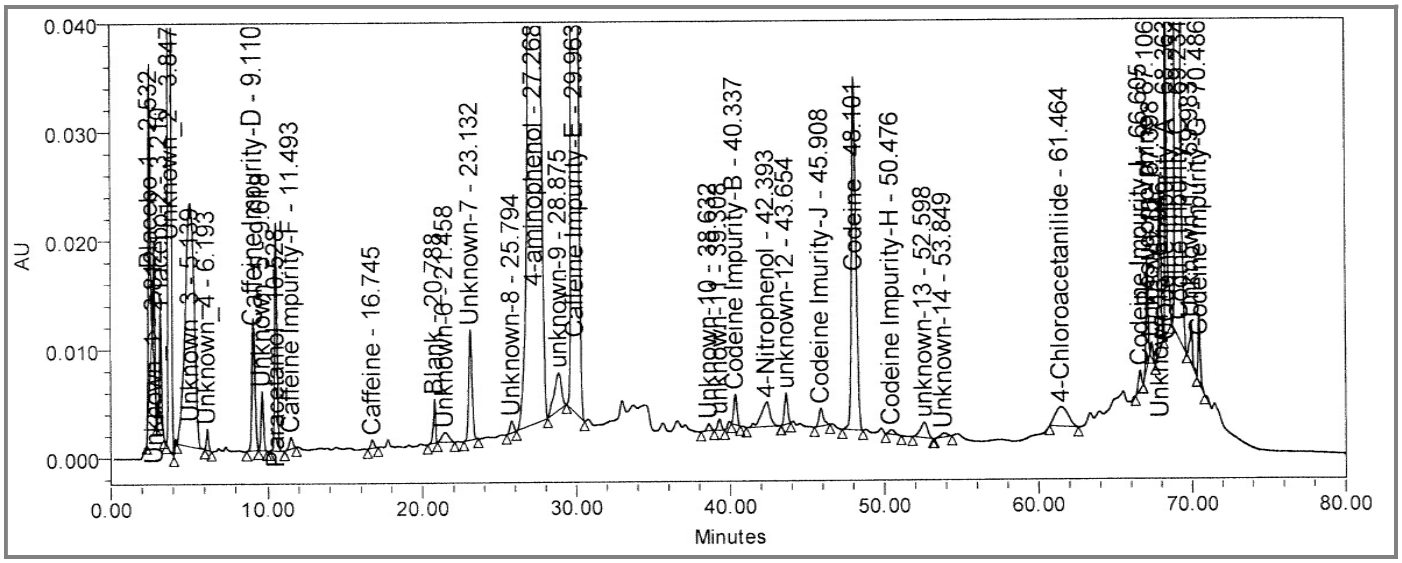

Fig 21. Reference chromatogram of base degradation.

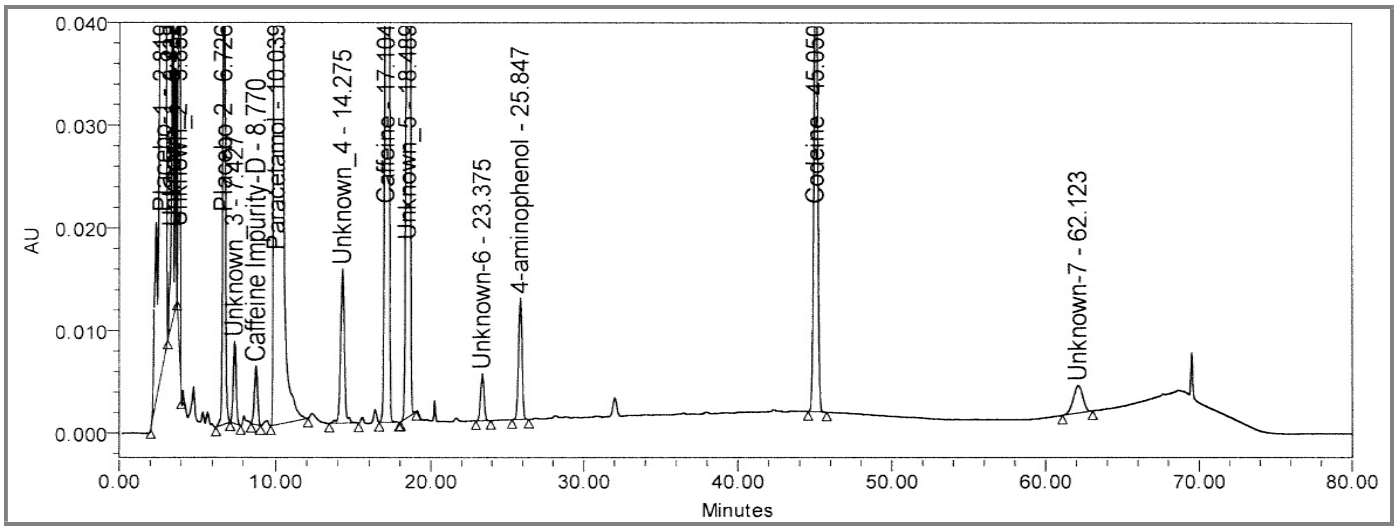

Fig 22. Reference chromatogram of peroxide degradation.

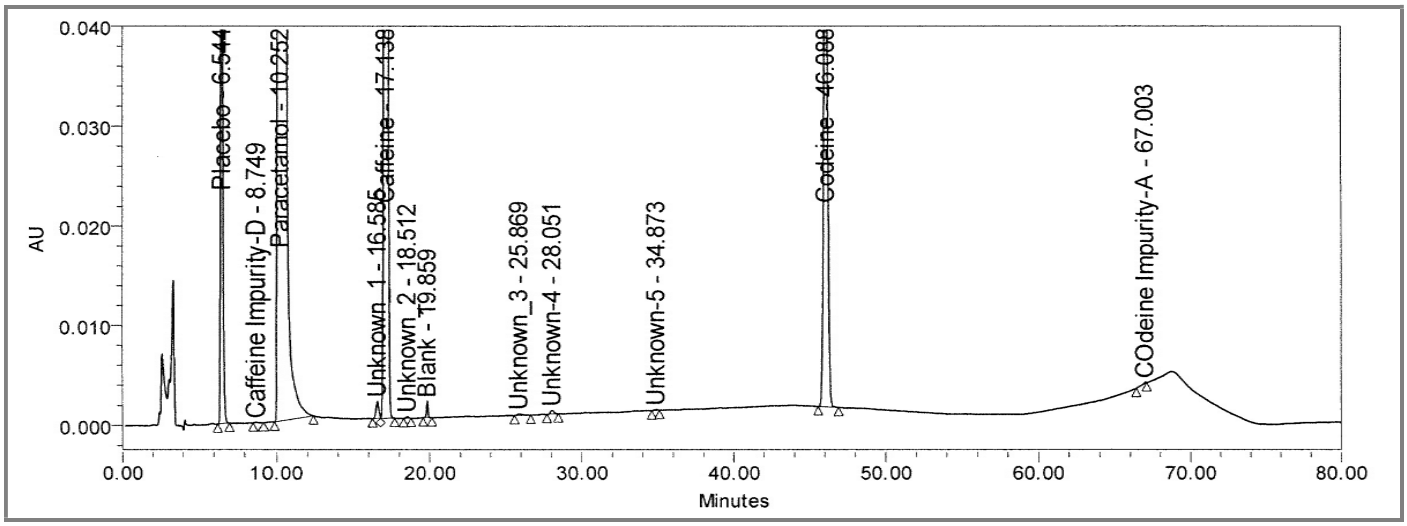

Fig 23. Reference chromatogram of thermal degradation.

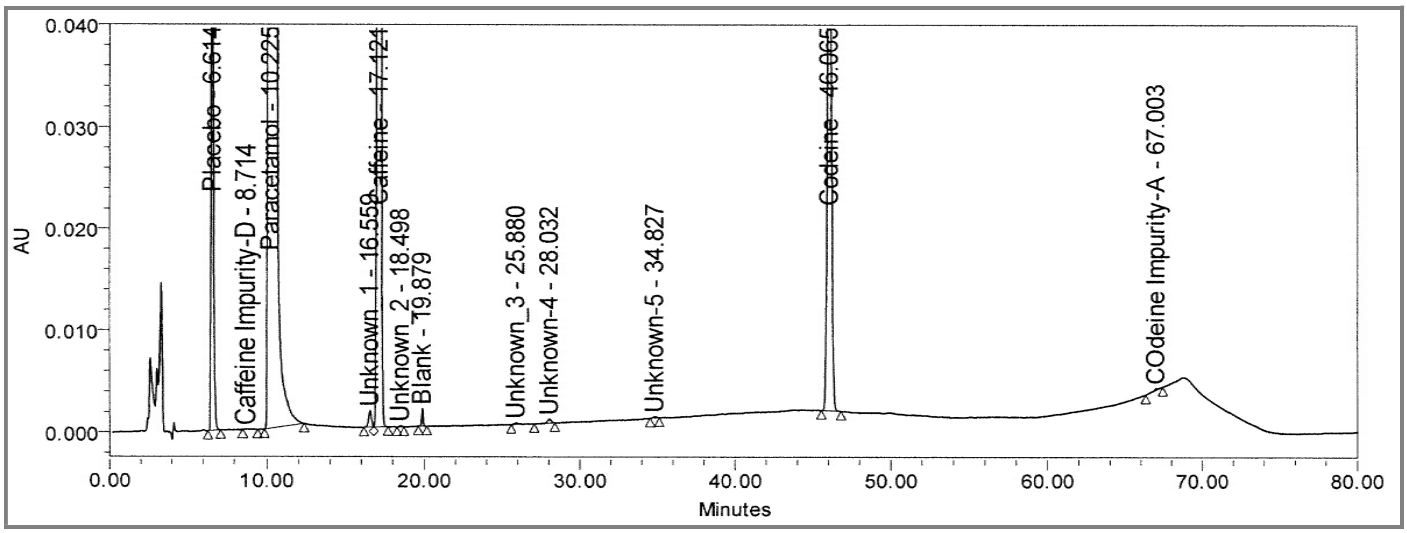

Fig 24. Reference chromatogram of UV degradation.

Table 11a. Forced degradation study (Paracetamol).

Sample Name	Sample area	% Assay	% Degradation	Purity angle	Purity Threshold
Control sample	77530381	-	-	12.889	15.001
TAcD (10N HCl/30 min)	68012950	87.7	12.3	13.209	15.001
TAcD (10N HCl/5 h)	32867	0.0	100.0	0.875	22.742
TAcD (10N HCl/2 h)	1504474	1.9	98.1	0.174	15.069
TAkD (5N NaOH/1 h)	66495103	85.8	14.2	10.241	15.013
TAkD (10N NaOH/3 h)	234203	0.3	99.7	1.020	18.552
TPD (30 % w/v H_2O_2/1 h)	57454227	74.1	25.9	10.358	15.015
TPD (30 % w/v H_2O_2/30 min)	56214182	72.5	27.5	10.848	5.163
TPD (30 % w/v H_2O_2/5 min)	66701544	86.0	14.0	12.609	15.001
TTD/100°C/1 Day	72989640	94.1	5.9	12.495	15.001
TUD/1 Day	71629657	92.4	7.6	13.596	15.001

TAcD - Tablets Acid degradation, TAkD - Tablets Alkali degradation, TPD - Tablets Peroxide degradation, TTD - Tablets Thermal Degradation, and TUD - Tablets UV Degradation.

Table 11b. Forced degradation study (Caffeine).

Sample Name	Sample area	% Assay	% Degradation	Purity angle	Purity Threshold
Control sample	4862922	-	-	1.877	15.002
TAcD (10N HCl/30 min)	4973054	102.3	-2.3	2.722	15.003
TAcD (10N HCl/5 h)	4787992	98.5	1.5	3.503	15.014
TAcD (10N HCl/2 h)	4708059	96.8	3.2	1.934	15.004
TAkD (5N NaOH/1 h)	4795653	98.6	1.4	2.338	15.044
TAkD (10N NaOH/3 h)	11395	0.2	99.8	3.515	35.408
TPD (30 % w/v H_2O_2/1 h)	5198334	106.9	-6.9	3.552	15.034
TPD (30 % w/v H_2O_2/30 min)	4976831	102.3	-2.3	4.306	1.884
TPD (30 % w/v H_2O_2/5 min)	4680004	96.2	3.8	0.608	15.002
TTD/100°C/1 Day	4936241	101.5	-1.5	2.471	15.001
TUD/1 Day	4733907	97.3	2.7	3.907	15.003

TAcD - Tablets Acid degradation, TAkD - Tablets Alkali degradation, TPD - Tablets Peroxide degradation, TTD - Tablets Thermal Degradation, and TUD - Tablets UV Degradation.

Table 11c. Forced degradation study (Codeine).

Sample Name	Sample area	% Assay	% Degradation	Purity angle	Purity Threshold
Control sample	1004668	-	-	0.093	15.028
TAcD (10N HCl/30 min)	994629	99.0	1.0	0.092	15.044
TAcD (10N HCl/5 h)	455005	45.3	54.7	0.120	15.438
TAcD (10N HCl/2 h)	632434	62.9	37.1	0.202	15.128
TAkD (5N NaOH/1 h)	912101	90.8	9.2	0.471	15.740
TAkD (10N NaOH/3 h)	666272	66.3	33.7	0.684	15.859
TPD (30 % w/v H_2O_2/1 h)	607514	60.5	39.5	9.027	15.833
TPD (30 % w/v H_2O_2/30 min)	121467	12.1	87.9	20.222	55.027
TPD (30 % w/v H_2O_2/5 min)	819315	81.6	18.4	0.259	15.069
TTD/100°C/1 Day	1031534	102.7	-2.7	0.134	15.020
TUD/1 Day	1006383	100.2	-0.2	0.101	15.037

TAcD - Tablets Acid degradation, TAkD - Tablets Alkali degradation, TPD - Tablets Peroxide degradation, TTD - Tablets Thermal Degradation, and TUD - Tablets UV Degradation.

Table 3. Method Precision Study (un-spiked sample).

Sample No.	4-AP	4-CA	CI-E	CoI-I	CoI-J	% SMUI	% TI
1	ND	ND	ND	ND	ND	0.005	0.005
2	ND	ND	ND	ND	ND	0.005	0.005
3	ND	ND	ND	ND	ND	0.005	0.005
4	ND	ND	ND	ND	ND	0.005	0.005
5	ND	ND	ND	ND	ND	0.005	0.005
6	ND	ND	ND	ND	ND	0.005	0.005
Mean	-	-	-	-	-	0.005	0.005
SD	-	-	-	-	-	0.000	0.000
% RSD	-	-	-	-	-	0.0	0.0

AP – Amino phenol, CA – Chloro Acetanilide, CI – Caffeine Impurity, CoI - Codeine Impurity, SMUI - Single max. unknown impurity and TI – Total Impurity.

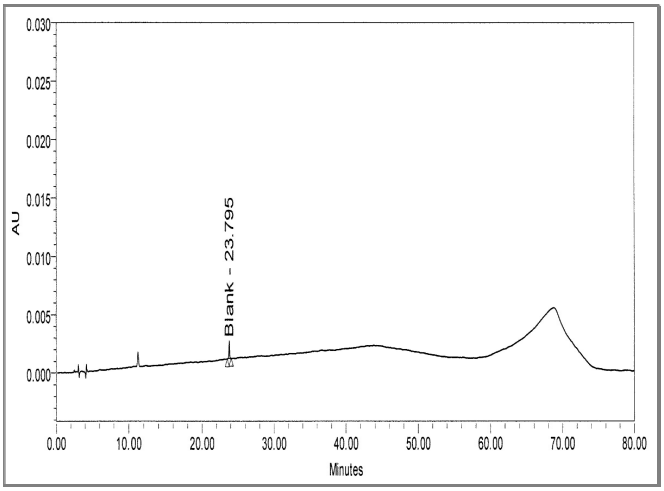

Fig 12. Reference chromatogram of blank.

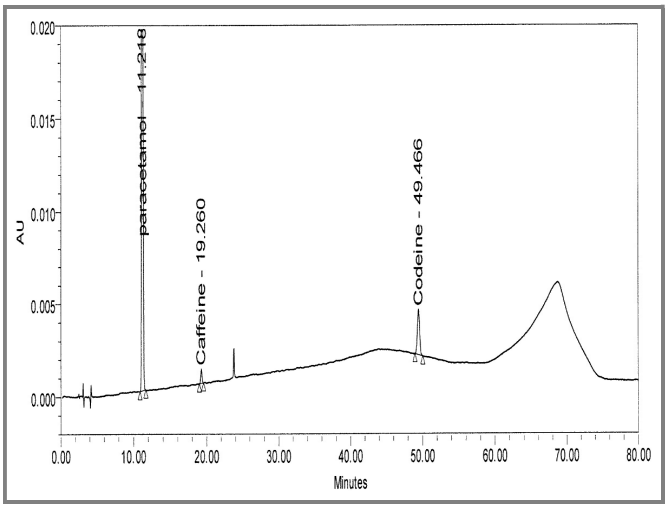

Fig 13. Reference chromatogram of standard solution.

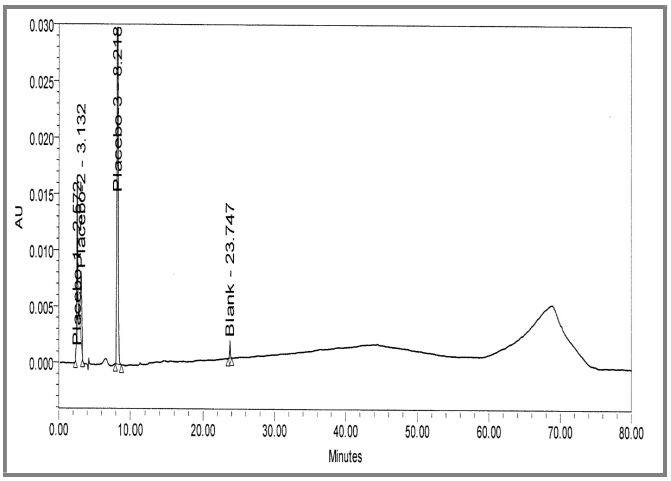

Fig 14. Reference chromatogram of placebo solution.

Calculate the percentage specified impurity for each preparation. Deduce % RSD for percentage specified impurity calculated for the six replicate preparations. The data obtained for the six sample preparations have been presented in Table 4 and Fig 16 for the chromatogram.

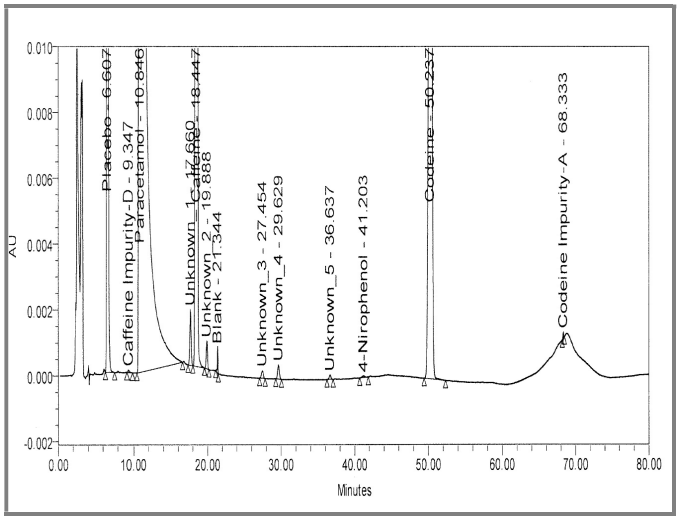

Fig 15. Reference chromatogram of sample solution.

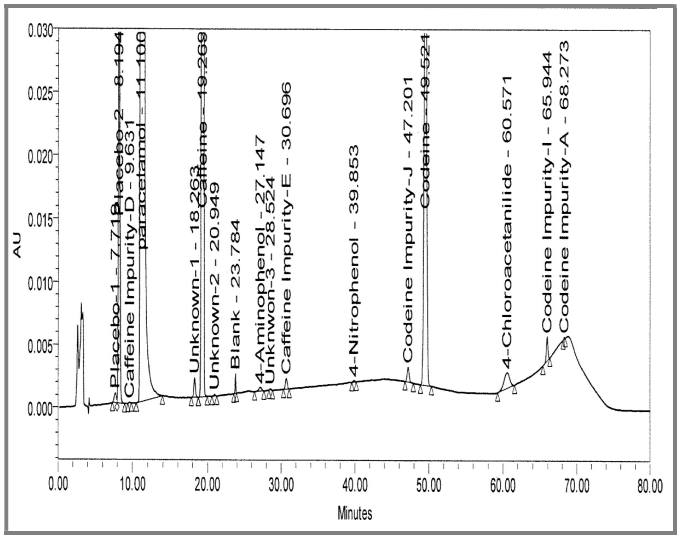

Fig 16. Reference chromatogram of spike sample solution.

Ruggedness (Unspike Sample):

The ruggedness of the method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot, and using a different HPLC system. The sample was prepared in six replicates and injected into the chromatograph. Calculate the percentage specified and unspecified impurity for each preparation. Deduce % RSD for percentage specified and % unspecified impurity. The data obtained for the six-sample preparative has been presented in Tables 5a to 5c.

Ruggedness (Spike Sample):

The ruggedness of the method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot, and using a different HPLC system.

Table 4. Method Precision Study (spiked sample).

Sample No.	4-AP	4-CA	CI-E	CoI-I	CoI-J	% SMUI	% TI
1	0.113	0.011	0.085	1.800	1.962	0.005	0.005
2	0.105	0.010	0.087	1.786	1.728	0.005	0.005
3	0.108	0.011	0.088	1.789	1.668	0.005	0.005
4	0.090	0.010	0.083	1.778	1.633	0.005	0.005
5	0.103	0.010	0.099	2.049	1.676	0.005	0.005
6	0.092	0.010	0.088	1.859	1.641	0.005	0.005
Mean	0.102	0.010	0.088	1.844	1.718	0.005	0.005
SD	0.009	0.001	0.006	0.105	0.124	0.000	0.000
% RSD	8.8	10.0	6.8	5.7	7.2	0.0	0.0

AP – Amino phenol, CA – Chloro Acetanilide, CI – Caffeine Impurity, CoI - Codeine Impurity, SMUI - Single max. unknown impurity and TI – Total Impurity.

Table 5a. Intermediate Method Precision Study (Unspike sample).

Sample No.	4-AP	4-CA	CI-E	CoI-I	CoI-J	% SMUI	% TI
1	ND	ND	ND	ND	ND	0.005	0.005
2	ND	ND	ND	ND	ND	0.005	0.005
3	ND	ND	ND	ND	ND	0.005	0.005
4	ND	ND	ND	ND	ND	0.005	0.005
5	ND	ND	ND	ND	ND	0.005	0.005
6	ND	ND	ND	ND	ND	0.005	0.005
Mean	-	-	-	-	-	0.005	0.005
SD	-	-	-	-	-	0.000	0.000
% RSD	-	-	-	-	-	0.0	0.0

AP – Amino phenol, CA – Chloro Acetanilide, CI – Caffeine Impurity, CoI - Codeine Impurity, SMUI - Single max. unknown impurity and TI – Total Impurity.

Table 5b. Precision & Intermediate comparison (Un-spike sample): SET-I & SET-II.

Sample ID#	% 4-Aminophenol		% 4-Chloro acetanilide		Caffeine Impurity-E		Codeine Impurity-J		Codeine Impurity-I	
	SET-I	SET-II	SET-I	SET-II	SET-I	SET-II	SET-I	SET-II	SET-I	SET-II
1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mean	-	-	-	-	-	-	-	-	-	-
SD	-	-	-	-	-	-	-	-	-	-
% RSD	-	-	-	-	-	-	-	-	-	-
Overall Mean	-		-		-		-		-	
Overall SD	-		-		-		-		-	
Overall % RSD	-		-		-		-		-	

Table 5c. Precision & Intermediate comparison (Un-spike sample): SET-I and SET-II.

Sample ID#	% Single max. unknown		% Total impurities	
	SET-I	SET-II	SET-I	SET-II
1	0.005	0.005	0.005	0.005
2	0.005	0.005	0.005	0.005
3	0.005	0.005	0.005	0.005
4	0.005	0.005	0.005	0.005
5	0.005	0.005	0.005	0.005
6	0.005	0.005	0.005	0.005
Mean	0.005	0.005	0.005	0.005
SD	0.000	0.000	0.000	0.000
% RSD	0.0	0.0	0.0	0.0
Overall Mean	0.005		0.005	
Overall SD	0.000		0.000	
Overall % RSD	0.0		0.0	

Table 6a. Intermediate Method Precision Study (spiked sample).

Sample No.	Sample No.	4-AP	4-CA	CI-E	CoI-I	CoI-J	% SMUI
1	0.091	0.012	0.098	1.715	1.586	0.005	0.005
2	0.087	0.012	0.098	1.706	1.622	0.005	0.005
3	0.092	0.012	0.098	1.706	1.602	0.005	0.005
4	0.086	0.012	0.096	1.712	1.584	0.005	0.005
5	0.085	0.012	0.097	1.687	1.597	0.005	0.005
6	0.090	0.012	0.098	1.684	1.590	0.005	0.005
Mean	0.089	0.012	0.098	1.702	1.597	0.005	0.005
SD	0.003	0.000	0.001	0.013	0.014	0.000	0.000
%RSD	3.4	0.0	0.8	0.8	0.9	0.0	0.0

AP – Amino phenol, CA – Chloro Acetanilide, CI – Caffeine Impurity, CoI - Codeine Impurity, SMUI - Single max. unknown impurity and TI – Total Impurity.

Table 6b. Precision & Intermediate precision comparison (spike sample) - SET-I & SET-II.

Sample ID#	% 4-Aminophenol		% 4-Chloro acetanilide		Caffeine Impurity-E		Codeine Impurity-J		Codeine Impurity-I	
	Set-I	Set-II	Set-I	Set-II	Set-I	Set-II	Set-I	Set-II	Set-I	Set-II
1	0.113	0.091	0.011	0.012	0.085	0.098	1.800	1.715	1.962	1.586
2	0.105	0.087	0.010	0.012	0.087	0.098	1.786	1.706	1.728	1.622
3	0.108	0.092	0.011	0.012	0.088	0.098	1.789	1.706	1.668	1.602
4	0.090	0.086	0.010	0.012	0.083	0.096	1.778	1.712	1.633	1.584
5	0.103	0.085	0.010	0.012	0.099	0.097	2.049	1.687	1.676	1.597
6	0.092	0.090	0.010	0.012	0.088	0.098	1.859	1.684	1.641	1.590
Mean	0.102	0.089	0.010	0.012	0.088	0.098	1.844	1.702	1.718	1.597
SD	0.009	0.003	0.001	0.000	0.006	0.001	0.105	0.013	0.124	0.014
% RSD	8.8	3.4	10.0	0.0	6.8	0.8	5.7	0.8	7.2	0.9
Overall Mean	0.095		0.091		0.093		1.773		1.657	
Overall SD	0.009		0.001		0.006		0.103		0.105	
Overall % RSD	9.5		9.1		6.5		5.8		6.3	

Prepare sample in six replicates as per the proposed method by spiking 4-aminophenol, 4-Chloroacetanilide, Caffeine Impurity-E, Codeine Impurity-I, Codeine Impurity-J at the specification level (0.01, 0.1, and 1.5 % with respect to sample concentration) and inject into the chromatograph. The % specified impurity for each preparation was calculated. The % RSD for the percentage specified impurity calculated for the six replicate preparations was deduced. The data obtained for the six sample preparations have been presented in Table 6a and 6b.

Linearity and range:

Standard Linearity Stock solutions containing Paracetamol, Caffeine, Codeine, 4-aminophenol, 4-Chloroacetanilide, Caffeine Impurity-E, Codeine Impurity-I, and Codeine Impurity-J were prepared. Linearity was determined by duplicate injections of 6 different concentrations (LOQ, 50, 100, 120, and 150 % of the target concentration).

The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate the coefficient of correlation, slope, and intercept. In general, a value of correlation coefficient (r2) > 0.99 is considered evidence of an acceptable fit for the data to the regression line.

The results obtained are shown in Table 7a to 8h and the data shows that the current method was linear for the eight analytes in the range specified above with a correlation coefficient of better than 0.99. The plots have been shown in Fig 4 to 11 and Fig 17 for the chromatogram.

Table 7a. Linearity of Paracetamol.

Level No.	Conc. Paracetamol (µg/ml)	Area Paracetamol
Level-1 LOQ	0.040	2309
Level-2 (50%)	10.020	454642
Level-3 (100%)	20.040	858312
Level-4 (120%)	24.048	1006151
Level-5 (150%)	30.060	1239885
Slope		41089.636
Intercept		20244.581
R^2		1.00

Table 7b. Linearity of 4-Aminophenol.

Level No.	Conc. 4-Aminophenol (µg/ml)	Area 4-Aminophenol
Level-1 LOQ	3.573	3276
Level-2 (50%)	5.105	3637
Level-3 (100%)	10.210	8134
Level-4 (120%)	12.252	9487
Level-5 (150%)	15.315	11146
Slope		715.292
Intercept		490.221
R^2		0.99

Table 7c. Linearity of 4-Chloroacetanilide.

Level No.	Conc. - 4-Chloroacetanilide (µg/ml)	Area - 4-Chloroacetanilide
Level-1 LOQ	0.336	22691
Level-2 (50%)	0.561	38110
Level-3 (100%)	1.121	68130
Level-4 (120%)	1.346	82751
Level-5 (150%)	105461	105461
Slope		60101.130
Intercept		2774.540
R^2		1.00

Table 7d. Linearity of caffeine.

Level No.	Conc. caffeine (µg/ml)	Area caffeine
Level-1 LOQ	0.240	2617
Level-2 (50%)	0.601	5180
Level-3 (100%)	1.201	10091
Level-4 (120%)	1.441	11749
Level-5 (150%)	14459	14459
Slope		7655.489
Intercept		728.267
R^2		1.00

Table 7e. Linearity of Caffeine Impurity-E.

Level No.	Conc. Caffeine Impurity-E (µg/ml)	Area Caffeine Impurity-E
Level-1 LOQ	0.337	4627
Level-2 (50%)	0.337	4750
Level-3 (100%)	0.674	8716
Level-4 (120%)	0.809	11725

Level-5 (150%)	1.012	13492
Slope		13449.080
Intercept		136.628
R^2		0.99

Table 7f. Linearity of Codeine.

Level No.	Conc. Codeine (µg/ml)	Area Codeine
Level-1 LOQ	0.311	2095
Level-2 (50%)	4.437	30222
Level-3 (100%)	8.873	55378
Level-4 (120%)	10.648	66052
Level-5 (150%)	81751	81751
Slope		6074.196
Intercept		1449.710
R^2		1.00

Table 7g. Linearity of Codeine Impurity-J.

Level No.	Conc. Codeine Impurity-J (µg/ml)	Area Codeine Impurity-J
Level-1 LOQ	0.356	3068
Level-2 (50%)	1.526	12141
Level-3 (100%)	3.051	22811
Level-4 (120%)	3.763	27409
Level-5 (150%)	4.577	34893
Slope		7367.465
Intercept		507.906
R^2		1.00

Table 7h. Linearity of Codeine Impurity-I.

Level No.	Conc. Codeine Impurity-I (µg/ml)	Area Codeine Impurity-I
Level-1 LOQ	0.386	2576
Level-2 (50%)	1.931	16107
Level-3 (100%)	3.862	30823
Level-4 (120%)	4.763	38864
Level-5 (150%)	5.793	48212
Slope		8326.234
Intercept		549.842
R^2		1.00

Table 8a. Range of 4-Aminophenol.

Injection #	LOQ level	Higher Conc. (150%)
1	3276	11386
2	3331	11557
3	3505	11123
4	2980	11684
5	3380	10221
6	3268	12255
Mean	3290	11371
SD	174.806	677.975
% RSD	5.3	6.0

Table 8b. Range of Paracetamol.

Injection #	LOQ level	Higher Conc. (150%)
1	2309	1223339
2	2237	1222988
3	2116	1222797
4	2492	1221091
5	2746	1224276
6	2385	1220801
Mean	2381	1222549
SD	174.806	677.975
% RSD	9.2	0.1

Table 8c. Range of 4-Chloroacetanilide.

Injection #	LOQ level	Higher Conc. (150%)
1	22691	102731
2	27281	100863
3	27278	99243
4	24905	96852
5	26116	103282
6	22595	100215
Mean	25144	100531
SD	2127.780	2358.477
% RSD	8.5	2.4

Table 8d. Range of caffeine.

Injection #	LOQ level	Higher Conc. (150%)
1	2617	13845
2	3025	14652
3	2865	14377
4	2645	14163
5	2863	13865
6	2861	14305
Mean	2813	14201
SD	154.332	311.855
% RSD	5.5	2.2

Table 8e. Range of Caffeine Impurity - E.

Injection #	LOQ level	Higher Conc. (150%)
1	4627	12882
2	4326	12766
3	4598	13147
4	3918	12999
5	5207	11892
6	4200	13161
Mean	4479	12808
SD	443.175	473.774
% RSD	9.9	3.7

Table 8g. Range of Codeine.

Injection #	LOQ level	Higher Conc. (150%)
1	2095	80220
2	2303	78958
3	2117	80210
4	1840	79708
5	2318	78401
6	2127	79929
Mean	2133	79571
SD	173.614	737.716
% RSD	8.1	0.9

Table 8f. Range of Codeine Impurity-J.

Injection #	LOQ level	Higher Conc. (150%)
1	3068	32385
2	2470	31619
3	3148	32904
4	2791	31693
5	2626	31826
6	2810	32158
Mean	2819	32098
SD	256.966	490.521
% RSD	9.1	1.5

Table 8h. Range of Codeine Impurity-I.

Injection #	LOQ level	Higher Conc. (150%)
1	2576	45910
2	2506	46185
3	2313	46929
4	2751	47371
5	2869	46112
6	2959	46526
Mean	2662	46506
SD	241.872	555.166
% RSD	9.1	1.2

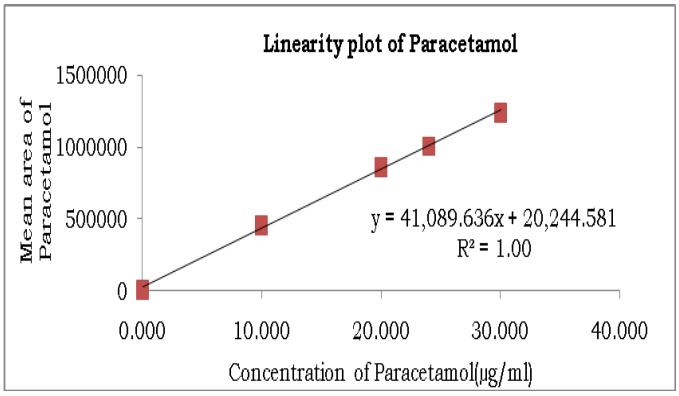

Fig 4. Linearity of Paracetamol.

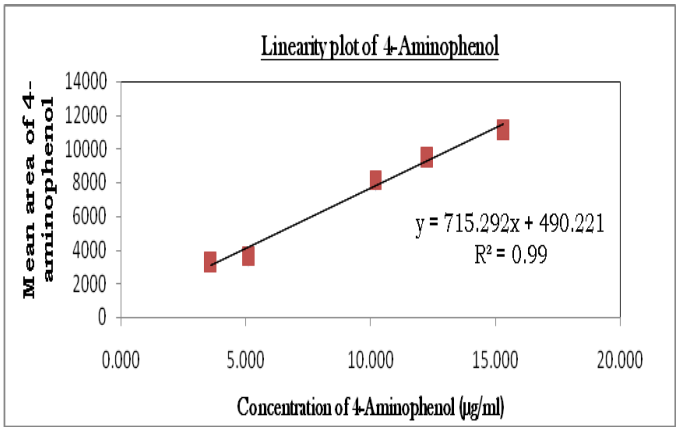

Fig 5. Linearity of 4-aminophenol.

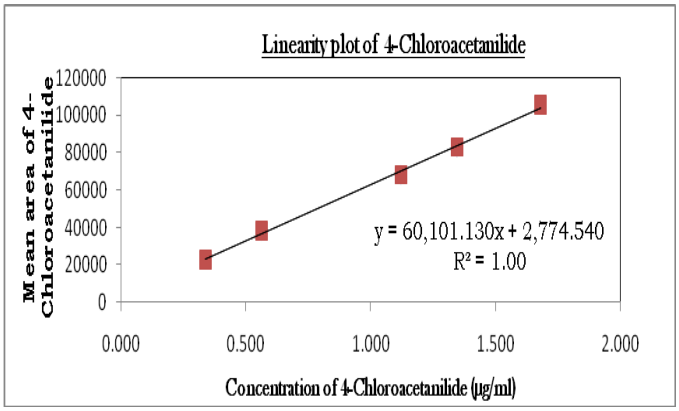

Fig 6. Linearity of 4-chloroacetanilide.

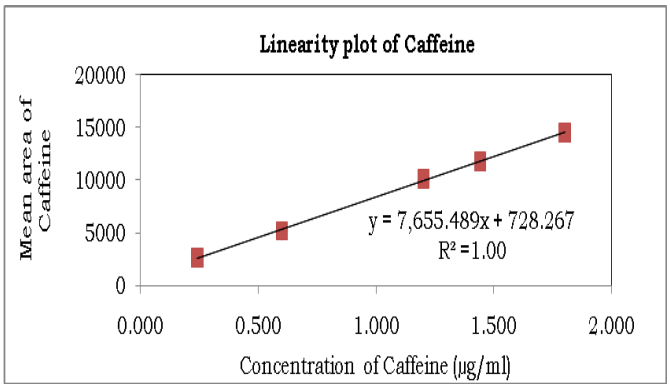

Fig 7. Linearity of Caffeine.

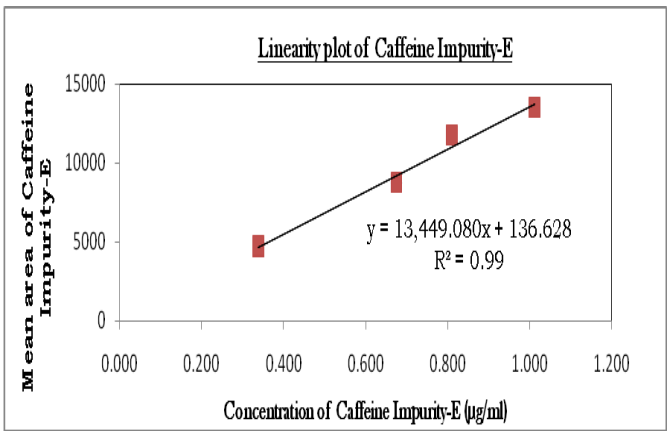

Fig 8. Linearity of Caffeine impurity-E.

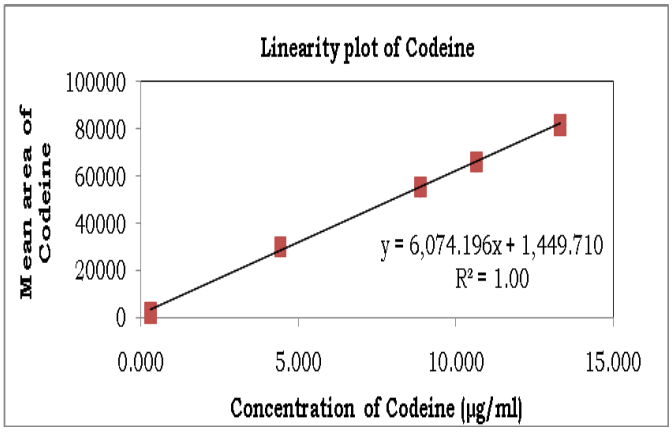

Fig 9. Linearity of Codeine.

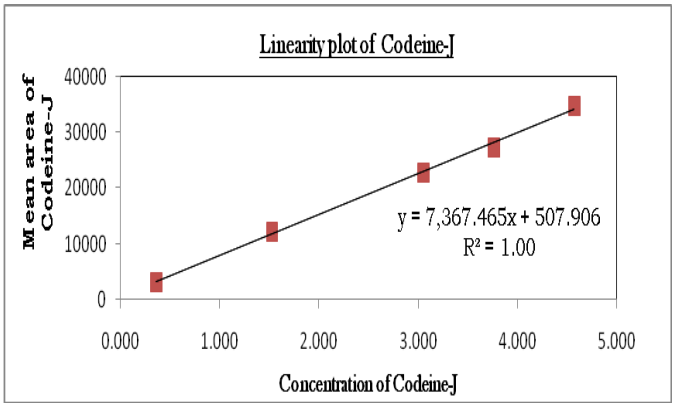

Fig 10. Linearity of Codeine Impurity-J.

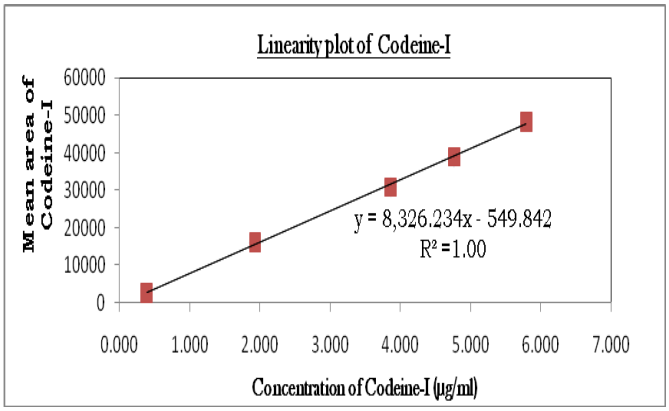

Fig 11. Linearity of Codeine Impurity-I.

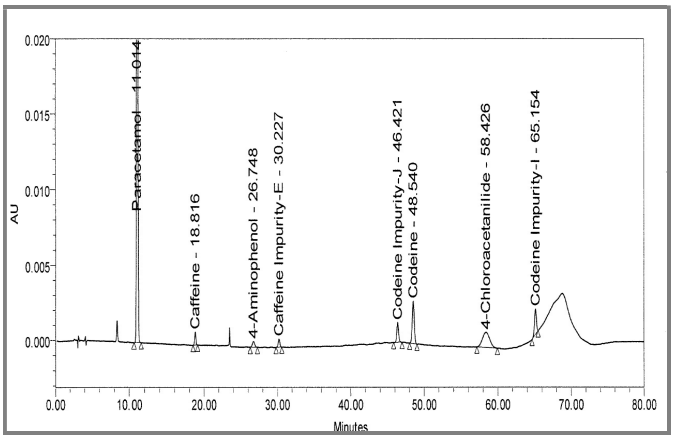

Fig 17. Reference chromatogram of linearity.

LOD and LOQ:

The LOQ and LOD solutions were injected at the predicted concentration 6 times and 3 times each respectively. The solutions having the calculated concentration were prepared by quantitative and stepwise dilutions of the linearity stock solution or any of the linearity solutions. The data obtained for the six preparations have been presented in Table 9a to 9h and Fig 18 and 19 for the chromatogram.

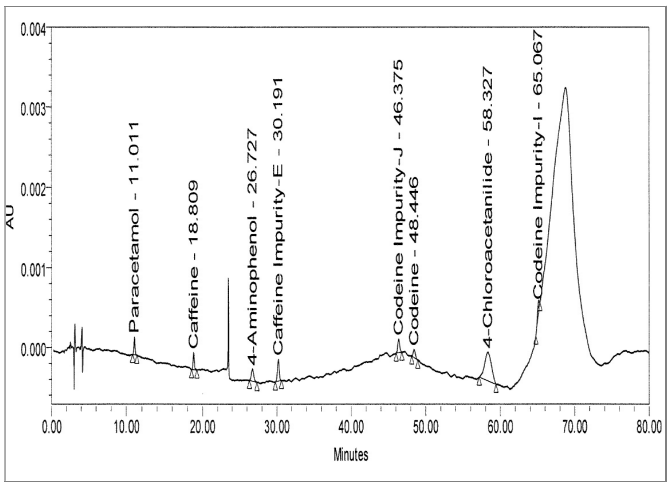

Fig 18. Reference chromatogram of LOQ solution.

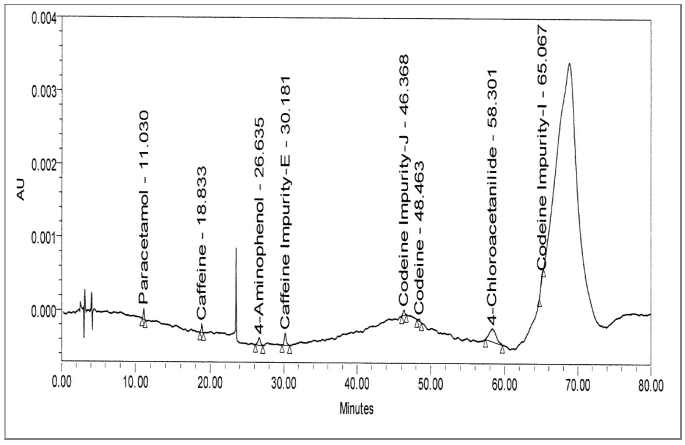

Fig 19. Reference chromatogram of LOD solution.

Table 9a. LOQ and LOD of Paracetamol.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.004 % 0.04 µg/ml		Conc. 0.002 % 0.02 µg/ml	
1	2309	29.00	1557	5.13
2	2237	20.15	1607	6.29
3	2116	28.93	1649	6.82
4	2492	45.66	-	-
5	2746	43.24	-	-
6	2385	28.44	-	-
Mean	2381	32.6	1604	6.08
SD	219.867	-	46.05	-
% RSD	9.2	-	2.9	-

Table 9b. LOQ and LOD of 4-Aminophenol.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.035% 3.573 µg/ml		Conc. 0.0175 % 1.75µg/ml	
1	3276	19.91	2589	3.64
2	3331	14.37	2244	3.79
3	3505	22.90	2250	4.23
4	2980	29.46	-	-
5	3380	28.87	-	-
6	3268	19.10	-	-
Mean	3290	22.4	2361	3.89
SD	174.806	-	197.477	-
% RSD	5.3	-	8.4	-

Table 9c. LOQ and LOD of 4-Chloroacetanilde.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.003 %, 0.3 µg/ml		Conc. 0.0015 % 0.15 µg/ml	
1	22691	47.41	11937	6.19
2	27281	34.90	10740	6.88
3	27278	20.83	13109	8.83
4	24905	69.98	-	-
5	26116	69.13	-	-
6	22595	42.35	-	-
Mean	25144	47.4	11929	7.30
SD	2127.78	-	1184.52	-
% RSD	8.5	-	9.9	-

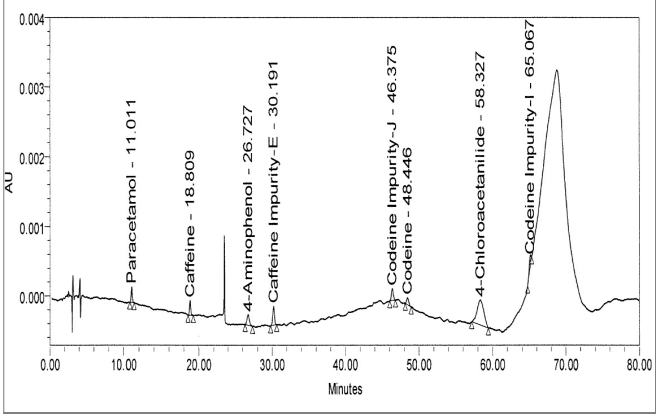

Fig 18. Reference chromatogram of LOQ solution.

Table 9d. LOQ and LOD of Caffeine.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.004 % 0.04 µg/ml		Conc. 0.002 % 0.02 µg/ml	
1	2617	28.60	1388	4.16
2	3025	22.97	1330	4.90
3	2865	32.72	1304	5.26
4	2645	45.04	-	-
5	2863	44.59	-	-
6	2861	30.85	-	-
Mean	2813	34.1	1341	4.77
SD	154.332	-	43.004	-
% RSD	5.5	-	3.2	-

Table 9e. LOQ and LOD of Caffeine Impurity-E.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.05 % 0.3 µg/ml		Conc. 0.025 % 0.15 µg/ml	
1	4627	35.81	3264	5.70
2	4326	24.14	2326	5.68
3	4598	35.36	2293	5.71
4	3918	48.51	-	-
5	5207	53.63	-	-
6	4200	32.98	-	-
Mean	4479	38.4	2628	5.70
SD	443.17	-	551.32	-
% RSD	9.9	-	21.0	-

Table 9f. LOQ and LOD of Codeine.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.2 % 0.32 µg/ml		Conc. 0.1 % 0.16 µg/ml	
1	2095	13.59	545	1.34
2	2303	9.38	856	2.07
3	2117	12.35	1586	3.45
4	1840	19.81	-	-
5	2318	19.83	-	-
6	2127	12.19	-	-
Mean	2133	14.53	996	2.29
SD	173.614	-	534.369	-
% RSD	8.1	-	53.7	-

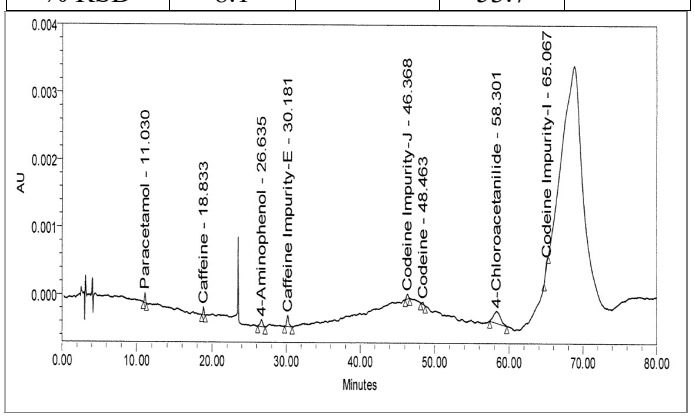

Fig 19. Reference chromatogram of LOD solution.

Table 9g. LOQ and LOD of Codeine Impurity-J.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.2 % 0.32 µg/ml		Conc. 0.1 % 0.16 µg/ml	
1	3068	22.23	1267	2.50
2	2470	13.81	1040	2.67
3	3148	22.74	1562	3.79
4	2791	32.60	-	-
5	2626	27.74	-	-
6	2810	21.64	-	-
Mean	2819	23.46	1290	2.99
SD	256.966	-	261.737	-
% RSD	9.1	-	20.3	-

Table 9h. LOQ and LOD of Codeine Impurity-I.

Inj #	LOQ		LOD	
	Area	S/N	Area	S/N
	Conc. 0.2 % 0.32 µg/ml		Conc. 0.1 % 0.16 µg/ml	
1	2576	21.75	1278	2.65
2	2506	14.63	1594	4.24
3	2313	20.83	1365	3.59
4	2751	33.75	-	-
5	2869	30.29	-	-
6	2959	22.32	-	-
Mean	2662	23.93	1412	3.49
SD	241.872	-	163.231	-
% RSD	9.1	-	11.6	-

Accuracy:

Accuracy study is to be conducted by spiking the known amount of 4-aminophenol, 4-Chloroacetanilide, Caffeine Impurity-E, Codeine Impurity-I and Codeine Impurity-J in the sample. The accuracy study was conducted in triplicate at four different levels (LOQ, 100 and 150 %) of the target concentration. The samples are to be analyzed as per methodology and percentage recovery at each spiked level was calculated. The data obtained for sample preparations have been presented in Table 10a to 10e and Fig 25 for the chromatogram.

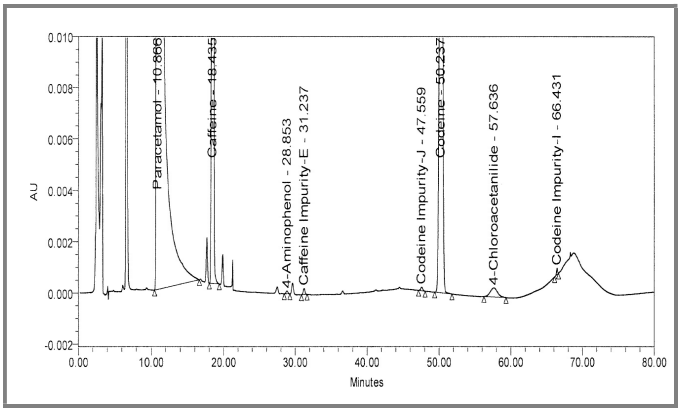

Fig 25. Reference chromatogram of accuracy.

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions.

CONCLUSION:

This intended study can be concluded as: the proposed method is economical, simple, ultra-fast, sensitive, and reliable. It is found to be accurate, precise, specific, stability-indicating, and rugged. All these parameters considered for verification meet the predefined acceptance criteria. So, the method is proposed for the quantitative estimation of related substances of Paracetamol, Caffeine, and Codeine in Paracetamol, caffeine, and Codeine Soluble Tablets 500/ 30/ 8 mg for intended applications.

ACKNOWLEDGEMENT:

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Table 10a. Accuracy of 4-Aminophenol.

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	% Recovery	% Recovery in each level	
1	I- (LOQ)	1	3.058	3.578	85.5	Avg.	83.4
2		2	2.919	3.578	81.6	SD	1.967
3		3	2.974	3.578	83.1	% RSD	2.4
7	II- (100 %)	1	9.753	10.223	95.4	Avg.	94.0
8		2	9.313	10.223	91.1	SD	2.512
9		3	9.766	10.223	95.5	% RSD	2.7
10	III- (150 %)	1	14.578	15.334	95.1	Avg.	96.6
11		2	14.998	15.334	97.8	SD	1.375
12		3	14.855	15.334	96.9	% RSD	1.4

Table 10b. Accuracy of 4-Chloroacetanilide

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	% Recovery	% Recovery in each level	
1	I- (LOQ)	1	0.406	0.336	120.8	Avg.	119.9
2		2	0.408	0.336	121.4	SD	2.043
3		3	0.395	0.336	117.6	% RSD	1.7
7	II- (100 %)	1	1.282	1.121	114.4	Avg.	114.5
8		2	1.291	1.121	115.2	SD	0.611
9		3	1.278	1.121	114.0	% RSD	0.5
10	III- (150 %)	1	1.904	1.682	113.2	Avg.	112.9
11		2	1.899	1.682	112.9	SD	0.252
12		3	1.895	1.682	112.7	% RSD	0.2

Table 10c. Accuracy of Caffeine Impurity-E.

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	% Recovery	% Recovery in each level	
1	I- (LOQ)	1	0.273	0.337	81.0	Avg.	80.4
2		2	0.271	0.337	80.4	SD	0.600
3		3	0.269	0.337	79.8	% RSD	0.7
7	II- (100 %)	1	0.630	0.674	93.5	Avg.	93.1
8		2	0.627	0.674	93.0	SD	0.404
9		3	0.625	0.674	92.7	% RSD	0.4
10	III- (150 %)	1	0.991	1.012	97.9	Avg.	99.3
11		2	1.024	1.012	101.2	SD	1.706
12		3	1.000	1.012	98.8	% RSD	1.7

Table 10d. Accuracy of Codeine Impurity-J.

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	% Recovery	% Recovery in each level	
1	I- (LOQ)	1	0.354	0.356	99.4	Avg.	100.8
2		2	0.373	0.356	104.8	SD	3.479
3		3	0.350	0.356	98.3	% RSD	3.5
7	II- (100 %)	1	2.926	3.051	95.9	Avg.	95.6
8		2	2.911	3.051	95.4	SD	0.289
9		3	2.910	3.051	95.4	% RSD	0.3
10	III- (150 %)	1	4.420	4.577	96.6	Avg.	96.7
11		2	4.448	4.577	97.2	SD	0.503
12		3	4.403	4.577	96.2	% RSD	0.5

Table 10e - Accuracy of Codeine Impurity-I

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	% Recovery	% Recovery in each level	
1	I- (LOQ)	1	0.303	0.354	85.6	Avg.	80.1
2		2	0.274	0.354	77.4	SD	4.734
3		3	0.274	0.354	77.4	% RSD	5.9
7	II- (100 %)	1	2.706	3.036	89.1	Avg.	90.1
8		2	2.767	3.036	91.1	SD	1.002
9		3	2.732	3.036	90.0	% RSD	1.1
10	III- (150 %)	1	4.017	4.554	88.2	Avg.	87.1
11		2	3.994	4.554	87.7	SD	1.436
12		3	3.893	4.554	85.5	% RSD	1.6

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