RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ACETAMINOPHEN AND BENZHYDROCODONE IN PHARMACEUTICAL FORMULATIONS

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Abstract: A RP-HPLC procedure is developed, validated and applied for simultaneous estimation of acetaminophen and benzhydrocodone in tablets. Procedure is based on separation and analysis of acetaminophen and benzhydrocodone in C18 column and 0.1MK_2HPO_4: methanol (60:40 v/v) mixture as stationary and mobile phase, respectively. The elution time values for acetaminophen and benzhydrocodone were 3.692 min and 4.956 min, respectively. Linear ranges for acetaminophen and benzhydrocodone are 162.5-487.5 µg/ml and 3.06-9.18 µg/ml, respectively. The values of sensitivity were 0.311 µg/ml (LOD) and 1.035 µg/ml (LOQ) for acetaminophen and 0.036 (LOD) µg/ml and 0.121 µg/ml (LOQ) for benhydrocodone. Validation parameters are tested using guidelines of ICH. The validation values obtained are well acceptable. The method proved as suitable procedure for assay of acetaminophen and benzhydrocodone in tablet dosage forms with good assay percent values.

Key words: acetaminophen, benzhydrocodone, Tablets RP-HPLC, K_2HPO_4: methanol, Validation.

INTRODUCTION

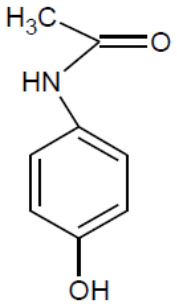
ACETAMINOPHEN N-(4-hydroxyphenyl) acetamide *.it comes under category of* Antipyretic, analgesic, Nonsteroidal Antiinflammatory. Acetaminophen also called as paracetamol, belongs to organic class of compounds, acetanilideThe mechanism is not clearly known. It is considered that acetaminophen primarily act in central nervous system, increasing the threshold of pain by blocking the isoforms of cyclooxygenase (COX-1, 2 and 3) enzymes which participates in prostaglandin synthesis

BENZHYDROCODONE 6,7-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6-yl benzoate it is a Opioid receptor antagonistThe prodrug, benzhydrocodone, is not active pharmacologically. It is metabolized to active hydrocodone by enzymes in the intestine . During this conversion benzhydrocodone undergoes O-demethylation, N-demethylation and 6-keto reduction. Hydrocodone acts like agonist for opioid receptors. It has more affinity for µ-opioid receptor. The correct analgesic mechanism is not known clearly

The aim to develop a method by RP-HPLC for the quantification of acetaminophen and benzhydrocodone simultaneously in tablet. The ICH guidelines following, validating the method for system suitability, linearity, precision, selectivity, sensitivity, accuracy, LOD&LOQ and robustness.

Work plan:

literature survey
Selecting the drug combination
Studying the properties of drug combination selected
Optimization of procedure using RP-HPLC technique
Validating the method developed
Results interpretation
Conclusion



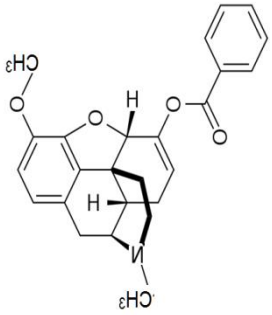


Fig 1. Structure of Acetaminophen **Fig 2. Structure of Benzhydrocodone**

MATERIALS AND METHODS

Materials:

- Reference drug material of acetaminophen and benzhydrocodone was collected from Sura Labs, Telangana, India.

I. Apadaz tablets: strength - 325 mg acetaminophen and 6.12 mg benzhydrocodone.
II. Methanol (HPLC grade) from Merck specialties Ltd, India
III. Dipotassium hydrogen phosphate (Analytical grade) from SD Fine-Chem Limited, India.

CHROMATOGRAPHIC CONDITIONS FOR ASSAY:

All analyses were done using an Waters Alliance HPLC system 2695 model, HPLC column Hibar C18 (250 × 4.6) mm, (5 µm), column oven and auto sampler were employed all through the analysis by HPLC. Solutions were injected using volumes of 20 µl at flow rate 1.0ml\min and a wavelength of 270 nm.

PREPARATION OF MOBILE PHASE

- 0.1M Dipotassium hydrogen phosphate and methanol were mixed at ratio 600 ml: 400 ml, respectively. pH is 4.5.

PREPARATION OF ACETAMINOPHEN AND BENZHYDROCODONE STANDARD SOLUTIONS:

325 mg of acetaminophen and 6.12 mg of benzhydrocodone were perfectly weighed and transferred to 100 ml volumetric flask then 30 ml mobile phase was added and sonicated 20 min. Mobile phase was further added to total the volume to 100 ml (Final Concentration - 3250 µg/ml acetaminophen and 61.20 µg/ml benzhydrocodone). This solution is acetaminophen and benzhydrocodone stock solution.

Acetaminophen and benzhydrocodone solutions for calibration curve are prepared by diluting 0.5, 0.75, 1.0, 1.25 and 1.5 ml of acetaminophen and benzhydrocodone stock solution to ten ml with mobile phase to get following concentrations:

162.5 µg/ml, 243.75 µg/ml, 325.0µg/ml, 406.25 µg/ml and 487.5 µg/ml – acetaminophen

3.06 µg/ml, 4.59 µg/ml, 6.12 µg/ml, 7.65 µg/ml and 9.18 µg/ml – benzhydrocodone

To study validation contents, acetaminophen and benzhydrocodone solution is made by diluting one ml of acetaminophen and benzhydrocodone stock to ten ml using mobile phase (final concentration: 325 µg/ml acetaminophen and 6.12 µg/ml benzhydrocodone).

PREPARATION OF ACETAMINOPHEN AND BENZHYDROCODONE TABLET SOLUTIONS:

Exactly weighed powdered tablet equal to acetaminophen 325 mg and benzhydrocodone 6.12 mg were perfectly weighed and transferred to 100 ml volumetric flask then 30 ml mobile phase was added and sonicated 20 min. Mobile phase was further added to total the volume to 100 ml (Concentration - 3250 µg/ml acetaminophen and 61.20 µg/ml benzhydrocodone). This solution of acetaminophen and benzhydrocodone tablet stock solution. For analysis, 1ml of acetaminophen and benzhydrocodone tablet stock solution is diluted to 10 ml using mobile phase (final concentration: 325 µg/ml acetaminophen and 6.12 µg/ml benzhydrocodone).

RESULT AND DISCUSSION

SYSTEM SUITABILITY:

Acetaminophen (325 µg/ml) and benzhydrocodone (6.12 µg/ml) solution injected five times. Criteria used for acceptance of system suitability are:

- Plate count - > 2000
- Resolution - > 2.0
- Peak tailing - ≤ 2.0
- RSD for peak area - ≤ 2.0

Table 1: Benzhydrocodone data during system suitability

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing
1	STD2	BENZHYDROCODONE	4.946	1013280	8071	6.80	1.36
2	STD2	BENZHYDROCODONE	4.944	1023461	8046	6.82	1.36
3	STD2	BENZHYDROCODONE	4.945	1014063	8022	6.82	1.36
4	STD2	BENZHYDROCODONE	4.948	1017599	8107	6.84	1.37
5	STD2	BENZHYDROCODONE	4.945	1016863	8067	6.84	1.36
Mean				1017053.2			
% RSD				0.4			

Table 2: Acetaminophen data during system suitability

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	STD2	ACETAMINOPHEN	3.685	3943605	11175	1.29
2	STD2	ACETAMINOPHEN	3.683	3974661	11233	1.28
3	STD2	ACETAMINOPHEN	3.683	3962823	11251	1.29
4	STD2	ACETAMINOPHEN	3.685	3963022	11259	1.29
5	STD2	ACETAMINOPHEN	3.683	3969532	11231	1.29
Mean				3962728.4		
% RSD				0.3		

SELECTIVITY:

Mobile phase blank, placebo blank, working solution (acetaminophen 325 µg/ml and benzhydrocodone - 6.12 µg/ml) and tablet solution (acetaminophen 325 µg/ml- benzhydrocodone - 6.12 µg/ml) were injected. Checked for interference peaks at the retention times of acetaminophen and benzhydrocodone. No interfering peaks were seen.

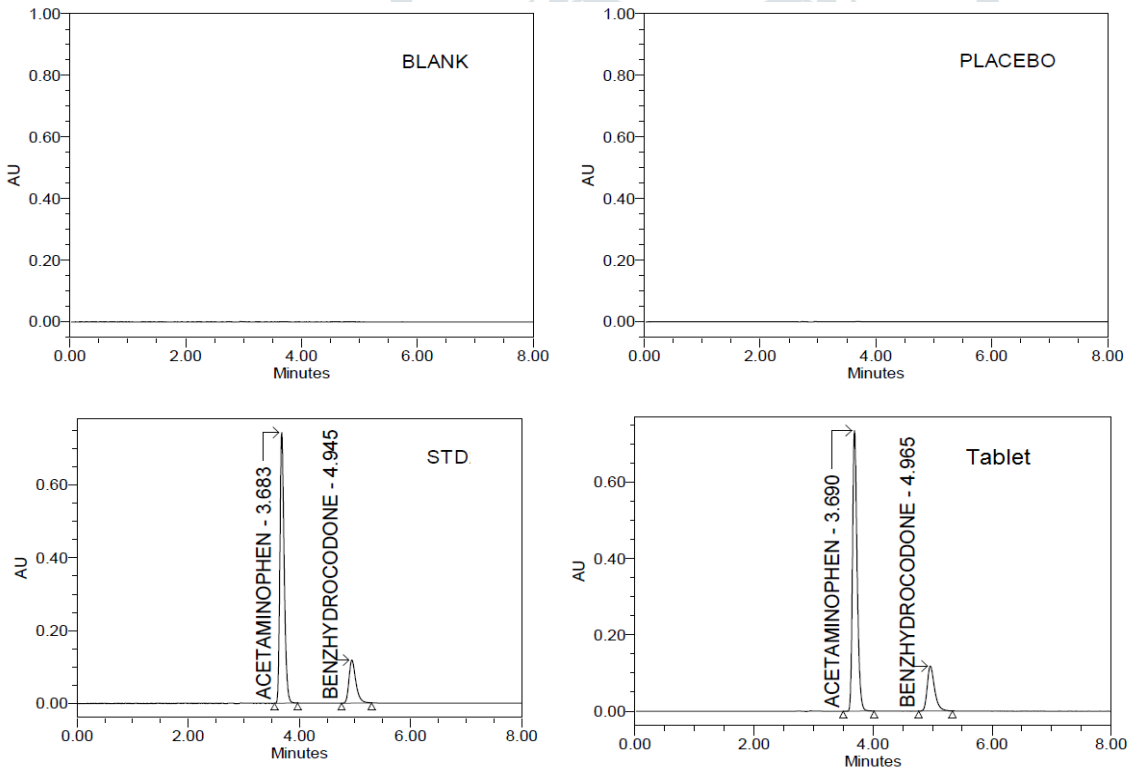


Figure 3: Selectivity chromatograms

LINEARITY:

The assay method linearity of acetaminophen and benzhydrocodone were determined in range from 50%, 75%, 100%, 125% and 150% proportional to concentration relative to standard concentration prescribed 325 µg/ml (acetaminophen) and 6.12 µgml (benzhydrocodone). The curves of acetaminophen and benzhydrocodone were linear over 162.5 – 487.5 µg/ml and 3.06 – 9.18 µg/ml, respectively and exhibited a good regression coefficient (R^2 = > 0.9990).

Table 3. Acetaminophen and benzhydrocodone linearity data

Conc %	Acetaminophen		Benzhydrocodone	
	Peak area	µg/ml	Peak area	µg/ml
50	1987907	162.5	508696	3.06
75	2979936	243.75	762481	4.59
100	3967196	325.00	1013570	6.12
125	4954061	406.25	1274750	7.65
150	5943775	487.5	1522204	9.18

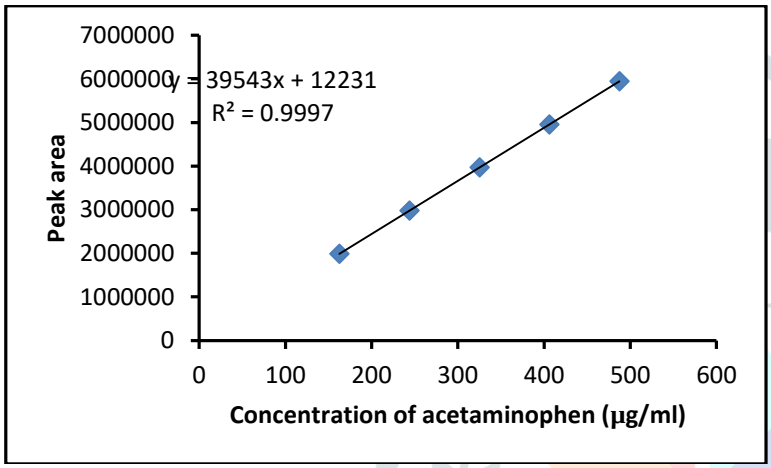


Figure 4. Acetaminophen linearity curve

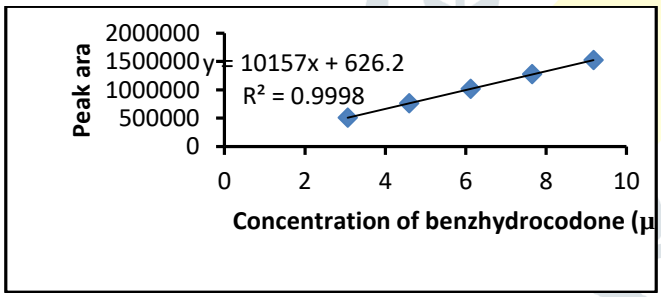
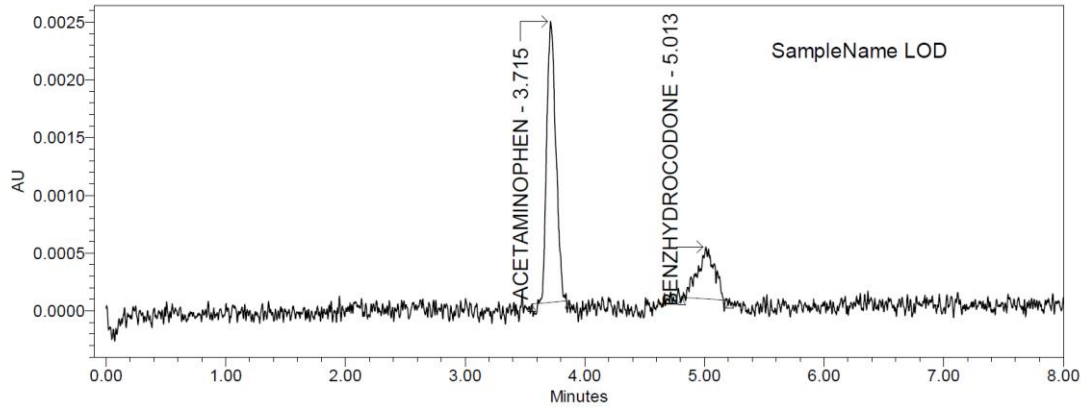
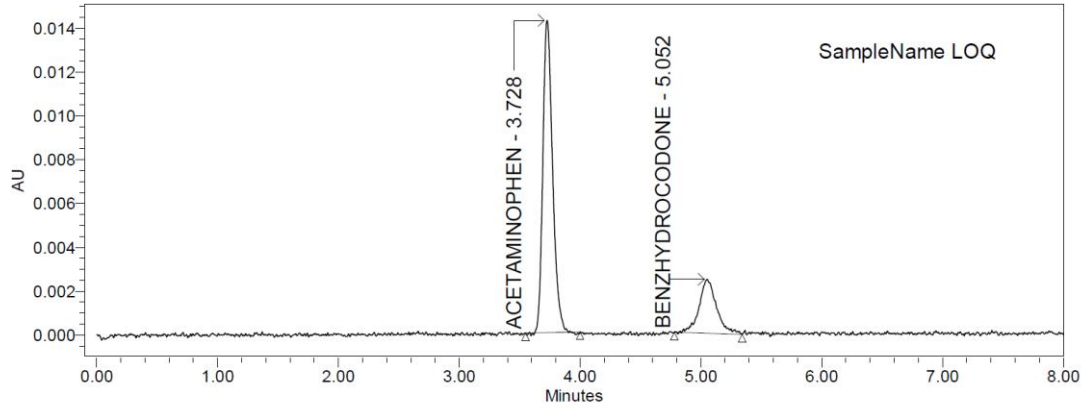


Figure 5. Benzhydrocodone linearity curve

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

Limit of detection (LOD) and limit of quantitation (LOQ) calculated as signal to noise ratio 3.1 and 10.1, respectively. LOD was 0.311 µg/ml for acetaminophen and 0.036 µg/ml for benzhydrocodone. LOQ was 1.035 µg/ml for acetaminophen and 0.121 µg/ml for benzhydrocodone.





Peak Name: BENZHYDROCODONE

	SampleName	Peak Name	RT	Area	s/n
1	LOD	BENZHYDROCODONE	5.013	4424	3.95
2	LOQ	BENZHYDROCODONE	5.052	24637	10.39

Peak Name: ACETAMINOPHEN

	SampleName	Peak Name	RT	Area	s/n
1	LOD	ACETAMINOPHEN	3.715	13204	3.50
2	LOQ	ACETAMINOPHEN	3.728	80207	10.29

Figure 6. LOD and LOQ chromatograms of acetaminophen and benzhydrocodone

PRECISION AND ACCURACY:

In this, standard solutions containing 325 µg/ml of acetaminophen and 6.12 µg/ml of benzhydrocodone were prepared, and injected 6 times into the HPLC system. Mean of peak areas and % RSD values of peak area and mean percent assay values were calculated to show precision and accuracy, respectively. Acceptable criteria are:

- Precision - %RSD ≤ 2.0
- Accuracy – percent assay 80-120%

Table 4: Acetaminophen and benzhydrocodone precision and accuracy results

Sample No.	Peak area Of acetaminophen	Peak area Of benzhydrocodone	Percent assay of acetaminphen	Percent assay of benzhydrocodone
1	3964203	1017773	99.74	99.67
2	3968526	1013573	99.85	99.26
3	3961837	1017638	99.68	99.66
4	3966868	1019607	99.8	99.85
5	3965045	1012037	99.76	99.11
6	3964217	1018343	99.74	99.73
Mean	**3965116**	**1016495.17**	**99.762**	**99.547**
SD	**2327.929**	**2981.652**	**0.058**	**0.292**
RSD	**0.059**	**0.293**	**0.058**	**0.290**

RECOVERY:

Recovery was tested by spiking 50, 100 and 150% of acetaminophen and benzhydrocodone standards to pre analyzed tablet solution in triplicates. The percent recovery was determined. An acceptance criterion is between 80% – 120% recovery value.

Table 5: Acetaminophen recovery results

Spiked Percent	Peak area of ACE	µg/ml of ACE added	µg/ml of ACE found	% of ACE Recovered	% Mean
50%	198577	162.500	162.32	99.89	99.94
50%	1983911	162.500	162.22	99.83	
50%	1989290	162.500	162.66	100.10	
100%	3965075	25.000	324.22	99.76	99.77
100%	3963716	25.000	24.11	9.72	
100%	3968061	325.000	324.46	9.83	
150%	5944048	487.500	486.03	9.70	.71
150%	5945780	87.500	86.18	.73	
150%	5944996	487.500	486.11	99.72	

Table 6: Benzhydrocodone recovery results

Spiked Percent	Peak area of BEN	µg/ml of BEN added	µg/ml of BEN found	% of BEN Recovered	% Mean
50%	508694	3.060	3.05	99.63	99.57
50%	508056	3.060	3.04	99.51	
50%	508299	3.060	3.05	99.56	
100%	1018778	6.120	6.11	99.77	99.59
100%	1012284	6.120	6.07	99.13	
100%	1019727	6.120	6.11	99.86	
150%	1525047	9.180	9.14	99.57	99.61
150%	152580	9.180	9.14	99.61	
150%	1526563	9.180	9.15	99.66	

ROBUSTNESS:

Small deliberate changes are made in the following:

Fig. 1. Ratio of methanol - changed by ±5%

Fig. 2. pH of buffer – changed by ± 0.2 units

Fig. 3. Flow rate - changed by ± 0.1 ml/min

Fig. 4. Column temperature - changed by ± 2 ºC;

Fig. 5. Wavelength – changed by ± 2 nm

In above changed conditions, acetaminophen (325 µg/ml) and benzhydrocodone (6.12 µg/ml) solution is injected. System suitability parameters determined. Criteria used for acceptance of system suitability are:

- Plate count - > 2000
- Resolution - > 2.0
- Peak tailing - ≤ 2.0
- RSD for peak area - ≤ 2.0

Table 7: Acetaminophen robustness

Conditions	Value Change	Tailing factor	Theoretical plate	Resolution
Column's temperature (°C)	23	1.30	9720	-
	27	1.29	10432	-
Flow rate run (ml/min)	0.9	1.31	11691	-
	1.1	1.32	12342	-
Mobile phase pH (units)	4.4	1.30	9720	-
	4.6	1.31	11691	-
Ratio of methanol (%)	30	1.29	11205	-
	40	1.29	11133	-
Wavelength (nm)	268	1.28	11210	-
	272	1.29	11218	-

Table 8: Benzhydrocodone robustness

Conditions changed	Changed value	Tailing factor	Theoretical plate	Resolution
Column's temperature (°C)	23	1.33	7214	6.54
	27	1.34	7768	6.86
Flow rate run (ml/min)	0.9	1.38	8478	7.16
	1.1	1.40	9180	7.46
Mobile phase pH (units)	4.4	1.33	7214	6.54
	4.6	1.38	8478	7.16
Ratio of methanol (%)	30	1.36	8073	6.85
	40	1.37	8023	6.82
Wavelength (nm)	268	1.37	7999	6.82
	272	1.37	8068	6.82

APPLICATION OF METHOD TO ASSAY ACETAMINOPHEN AND BENZHYDROCODONE IN TABLETS:

The content of acetaminophen and benzyhydrocodone was determined in Apadaz tablets (strength - 325 mg acetaminophen and 6.12 mg benzhydrocodone) by proposed method. The assay percent (nearer to 100%) and relative standard deviation (less than 2%) values are acceptable

Table 9: Assay of acetaminophen and benzhydrocodone in tablet

Drug content in tablet (mg)	Drug determined (µg/ml)	Drug Assayed (%)	Statistical assessment
Acetaminophen			
325	324.81	99.94	**Mean:** 99.81%
325	324.25	99.77	**RSD:** 0.120%
325	324.06	99.71	-
Benzhydrocodone			
6.12	6.094	99.57	**Mean:** 99.59%
6.12	6.095	99.59	**RSD:** 0.020%
6.12	6.096	99.61	-

CONCLUSION

Acetaminophen and benzhydrocodone were simultaneously separated and quantified successfully in the tablets using the developed RP-HPLC method with good precision and accuracy. The RP-HPLC method has adequate sensitivity and selectivity

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