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Analytical Method Development and Validation for the Simultaneous Estimation of Acetaminophene and oxycodone by RP-HPLC Method in Bulk and Pharmaceutical Dosage Form

V. Hari Baskar*, V. Pushpalatha, M. Gobinath, Ramesh Dhani

Department of Pharmaceutical Analysis, Ratnam Institute of Pharmacy, Pidathapolur, Nellore, Andhra Pradesh, India

A B S T R A C T

The Present work was to develop a simple, fast, accurate, precise, reproducible, Reverse Phase High Performance Liquid Chromatographic Method for simultaneous estimation of Acetaminophine and Oxycodone in pure drug form. Chromatographic separation was done using kromosil C_{18} Column (250mm x 4.6mm)5µg. with mobile phase consisting Phosphate buffer: Methanol P^H 2.5 (65:35 v/v) P^H was adjusted with ortho phosphoric acid and flow rate was adjusted to 1.0 ml/min and detection wavelength at 254nm. The retention times of acetaminophene Oxycodone was found to be2.589 ± 0.004 min and 3.711 ± 0.005 min. The proposed method has been validated for accuracy, precision, linearity, robustness and range were within the acceptance limit according to ICH guidelines. Linearity for Acetaminophene and Oxycodone was found in range 20 to 60 µg/ ml and 10 to 30µg/ml and correlation coefficient was found to be 0.997 and 0.999% RSD for intermediate precision was found to be within the limits for Acetaminophene and Oxycodone. Recovery for Acetaminophene and Oxycodone was found to be 99.3% to 99.3% respectively. The method was found to be robust even by change in the mobile phase ±5% and in less flow condition. The developed method can be successfully employed for the routine analysis of Acetaminophene and Oxycodone in API and Pharmaceutical dosage forms.

Keywords: Acetaminophene, Oxycodone, RP-HPLC, Method development, Validation**.**

A R T I C L E I N F O

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***Corresponding Author**
V. Hari Baskar
Department of Pharmaceutical Analysis,
Ratnam Institute of Pharmacy,
Nellore, Andhra Pradesh, India
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1. Introduction

Analytical methods: Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness [1,2]. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available

Description of the Various Analytical Methods

Titrimetric and gravimetric method of analysis is suitable when the sample is present in pure form or when no interference is observed in the mixture with other materials [3]. Ultraviolet and visible spectrometric method is suitable when no Interference is observed in the mixture [4]. HPLC and GC methods are more advantageous than the above due to their capability in separating organic mixtures and quantitative estimations. AAS is used mainly for quantitative estimation in ppm and ppb levels of elements Infra-red spectroscopy though mainly used for qualitative analysis can be used for quantitative estimation also. Out of all the above methods, thin layer chromatography plays a very important role in analysis due to its adaptability, flexibility, and cost and time. It can be used both for qualitative and quantitative determination. After separation spots can be scanned with the help of a scanner and quantitative measurement can be made [5, 6].

Chromatography:

Chromatography is a technique used in analytical chemistry to separate and identify components of mixtures. The name comes from the Greek term for "color writing" because this method was originally used to separate colored samples. The advent of high-performance liquid chromatography (HPLC).in this system pressure is applied to the column, forcing the mobile phase through at much higher rate[7]. The pressure is applied using a pumping system. The action of the pump is critical, since it must not pulsate and mix up the sample being separated in the solvent, causing it to lose resolution [8]. Development of pumps has proceeded quite quickly over the last several years, and now it is possible to achieve good resolution under the conditions required for HPLC [9].

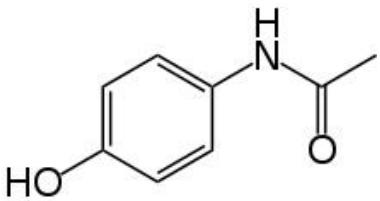

Figure 1: Acetaminophene

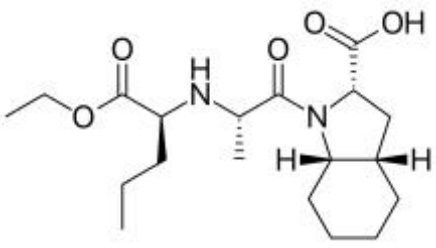

Figure 2: Oxycodone

2. Materials and Methods

Apparatus

The instrument used for the study was HPLC Autosampler. Separation module2695, PDA 996 diode array detector. Empower-software version-2.

Reagents and Materials

The solvents used were Methanol, Ortho phosphoric acid, Triethyl amine, Potassium dihydrogen ortho phosphate and Water [10].

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected [11].

Standard solutions of Acetaminophen and Oxycodone were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 254 nm was selected as the detection wavelength for the present study [12].

Selection of mobile phase

Initially the mobile phase tried was methanol and water, methanol and Methanol, buffer and water in various proportions. Finally, the mobile phase was optimized to Buffer: Methanol in proportion 65:35 v/v respectively [13].

Optimization Chromatographic trials for Simultaneous Estimation of Acetaminophene and Oxycodone by RP-HPLC.

Optimization Chromatographic conditions

Column : kromosil $C_{18\ Column}$ (250mm x 4.6mm) 5µg.
Mobile phase ratio: Phosphate buffer: Methanol P^H 2.5 (65:35 v/v)

Detection wavelength	:	254 nm
Flow rate	:	1.0ml/min
Injection volume	:	20µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	10min
Retention time	:	2.605 and 3.781 mins

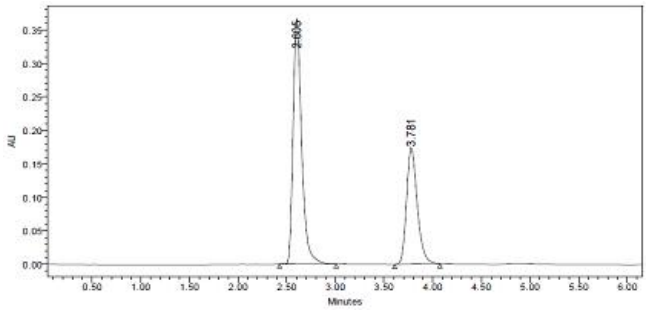

Figure 1: Optimization Chromatogram

Observation: The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

Preparation of phosphate buffer: About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and pH 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon

membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution [14].

Preparation of mobile phase

Mobile phase consist of buffer: Methanol of P^H 2.5 (35:65) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter [15].

Acetaminophene and Oxycodone standard preparations

Weigh accurately 10 mg Acetaminophen Working Reference Standard and 15mg of Oxycodone Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution) Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluents [16, 17].

Sample solutions preparation

10 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 10 tablets was transferred into a 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution). Further pipette 0.25ml of Acetaminophen and Oxycodone of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluents

3. Results and discussion

Method Validation Parameters

1. Specificity: ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present [18]. Typically this might include impurities, degradants, matrix.

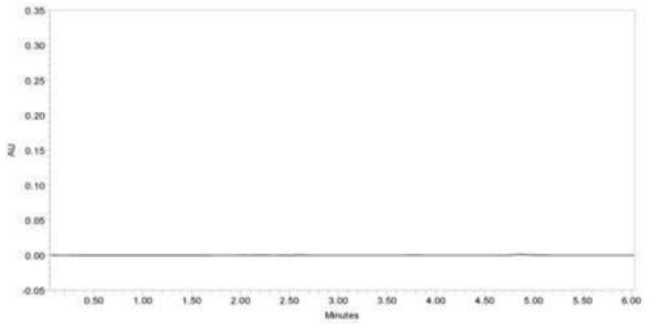

Figure 3: Chromatogram of Blank

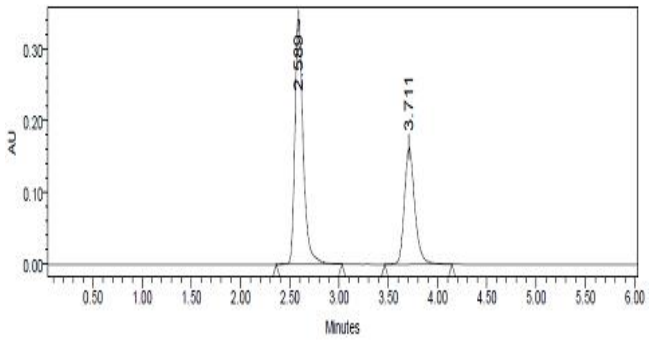

Figure 4: Chromatogram of Sample

2. Linearity and Range

Preparation of stock solution:

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [19]. Serial dilutions of Acetaminophen and Oxycodone (20-60µg/ml and 10-30 µg/ml) were injected into the column and detected at a wavelength set at 254 nm. The calibration curve was obtained by plotting the concentration vs. peak area.

4. Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

- Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.
- Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively [20].

Acceptance criteria: The mean % recovery of the Acetaminophen and Oxycodone at each level should be not less than 95.0% and not more than 105.0%.

5. Precision

The precision of the method was demonstrated by intra-day and inter-day precision studies. Intra-day studies were performed by injecting three (3) repeated injections within a day. Peak area and %RSD were calculated and reported. The chromatograms of intra-day precision studies were shown. Inter-day precision studies, was done by injecting three (3) repeated injections for three consecutive days. Peak area and %RSD were calculated and reported.

Method Precision:

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions. The % RSD of peak areas of six samples was calculated. The method precision was performed on Acetaminophen and Oxycodone formulation. The % RSD of the assay value for six determinations should not be more than 2.0%.

Intermediate Precision:

Intermediate precision of the analytical method was determined by performing method precision on another day by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and mean %assay value, standard deviation & %RSD was calculated

Validation of the Method

Linearity: The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Serial dilutions of Acetaminophen and Oxycodone (20-60µg/ml and 10-30 µg/ml) were injected into the column and detected at a wavelength set at 254 nm. The calibration

curve was obtained by plotting the concentration vs. peak area.

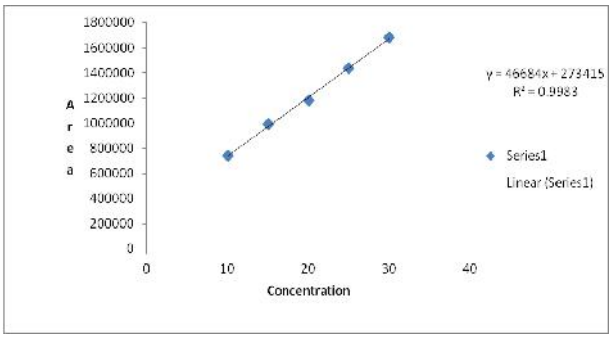

Figure 5: Calibration graph of Acetaminophene

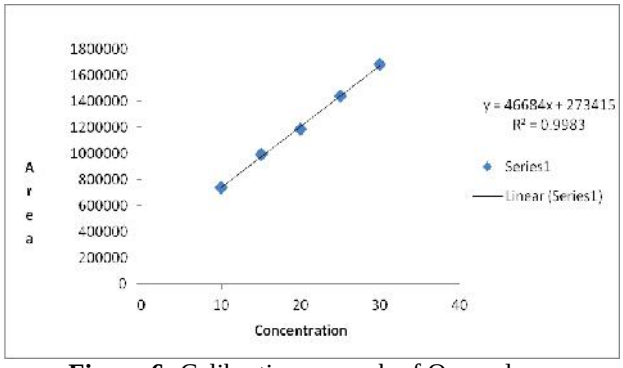

Figure 6: Calibration ganraph of Oxycodone

Accuracy: The accuracy was assessed by using a minimum of three different concentrations of standards, for these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively. Acceptance criteria: The mean % recovery of the Acetaminophen and Oxycodone at each level should be not less than 95.0% and not more than 105.0%.

Detection limit (LOD): The LOD was performed for Acetaminophene and Oxycodone was found to be 0.001 and 0.005 respectively.

Quantitation Limit (LOQ): The LOQ was performed for Acetaminophene and Oxycodon was found to be 0.004 and 0.015 respectively.

4. Conclusion

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Acetaminophene and Oxycodone in tablet dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. From literature review and solubility analysis initial chromatographic conditions Mobile phase ortho phosphoric acid buffer: Methanol 65:35 were set (Buffer P^H 2.45 adjusted with Triethyl amine), Kromosil C 18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 254 nm. As the methanol content was increased Acetaminophene and Oxycodone got eluted with good peak symmetric properties. The retention times for Acetaminophene and Oxycodone was found to be 2.589 min and 3.711 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to150 % levels, R^2 value was found to be as 0.999.By using above method assay of marketed formulation was carried out, 100.7% was present. Full length method was not performed; if it is done this method can be used for routine analysis of Acetaminophene and Oxycodone.

Table 1: Calibration data of Acetaminophene and Oxycodone

Sample ID	Acetaminophen		Oxycodone	
	Conc.(mcg/ml)	Area	Conc. (mcg/ml)	Area
20% of operating conc.	20	1224140	10	740046
40% of operating conc.	30	1595681	15	990204
60% of operating conc.	**40***	**1992966**	**20***	**1183023**
80% of operating conc.	50	2356546	25	1439886
100% of operating conc.	60	2797214	30	1682302
Correlation Coefficient			0.999	

Table 2: Accuracy results for Acetaminophene

Sample Id	Conc. found (µg/ml)	Conc. Obtained (µg/ml)	% Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		**%RSD= 0.505**
50%	5	4.96	99.2	99.73	
50%	5	4.99	99.8		
100%	10	9.95	99.5		**%RSD=0.66**
100%	10	9.87	98.7	98.8	
100%	10	9.82	98.2		
150%	15	14.64	97.6		**%RSD=1.45**
150%	15	14.76	98.4	98.8	
150%	15	15.06	100.4		

Table 3: Accuracy results for Oxycodone

Conc (µg/ml)	Concn Obtained(µg/ml)	% Recovery of drug	Mean accuracy	%RSD
5	4.92	98.0		
5	4.96	99.2		
5	5.02	100.4	**99.2**	**1.2**
10	9.95	99.5		
10	9.94	99.4		
10	9.98	99.8	**99.5**	**0.2**
15	14.78	98.6		
15	14.94	99.6	**99.0**	**0.530**
15	14.83	98.8		

Table 4: Robustness data for Acetaminophene

Std. Replicate	Variation in flow rate		Variation in Mobile phase composition	
	Flow Rate 0.8ml/min	Flow Rate 1.2ml/min	Buffer: Methanol (40:60)	Buffer: Methanol (30:70)
1	2492492	1676589	1951632	1979168
2	2495874	1675428	1954783	1967452
Mean	2494183	1676009	1953208.0	1973310
SD	2391.4	820.9	2228.0	8284.46
%RSD	0.09	0.04	0.11	0.4
Retention time	3.150	2.168	2.618	2.572
Tailing factor	1.4	1.3	1.3	1.3
Theoretical plates	5752	4207	4577	4476

Table 5: Robustness data for Oxycodone

Parameter	Variation in flow rate		Variation in Mobile phase composition	
Standard	Flow Rate 0.8ml/min	Flow Rate 1.2ml/min	Buffer: Methanol (40:60)	Buffer: Methanol (30:70)
1	1500192	100524	1196996	1153397
2	1500426	100468	1198547	1154782
Mean	1500309	100496	1197772	1154090
SD	165.5	39.59	1096.2	979.34
%RSD	0.01	0.03	0.09	0.08
Retention time	4.674	3.121	4.394	3.331
Tailing factor	1.2	1.2	1.2	1.2
Theoretical plates	7187	5412	6498	6471

Table 6: Method Precision data for Acetaminophen & Oxycodone

S.No	Concentration (µg/ml)	Acetaminophen		Oxycodone	
		Retention time (Rt)	Peak Area	Retention time (Rt)	Peak Area
1	40 & 20	2.586	2010800	3.713	1184689
2	40 & 20	2.588	2002956	3.714	1188199
3	40 & 20	2.590	2012800	3.734	1195842
4	40 & 20	2.590	2005243	3.737	1184210
5	40 & 20	2.591	2011092	3.741	1198327
6	40 & 20	2.589	2011098	3.740	1198320
Avg			2008998		1191598
SD			3920.9		6668.5
%RSD			**0.19**		**0.55**

Table 7: Intermediate precision data for Acetaminophene and Oxycodone

S.No.	Conc. (µg/ml)	Intermediate Precision			
		Acetaminophen		Oxycodone	
		Retention time	Peak Area	Retention time	Peak Area
1	40&20	2.591	2005053	3.741	1183951
2	40&20	2.590	2007362	3.734	1184689

3	40&20	2.590	2007473	3.737	1186232
4	40&20	2.586	2009153	3.714	1186406
5	40&20	2.583	2012800	3.713	1188564
6	40&20	2.590	2012785	3.737	1187621
Avg			2009104		1186244
SD			3140.6		1730.9
%RSD			**0.15**		**0.14**

Table 8: LOD and LOQ Data of Acetaminophen and Oxycodone

Acetaminophen			**Oxycodone**		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
40	2004682	S = 39092 c = 618048 LOD: 0.001µg/ml LOQ: 0.004µg/ml	20	1184227	S = 39092 c =369381 LOD:0.005 µg/ml LOQ: 0.015µg/ml
40	2004587		20	1186425	

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