AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Related Substances of Acetaminophen In Acetaminophen, Dextromethorphan HBr and Doxylamine Succinate Soft Gelatin Capsules by Using RP-HPLC Method

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ABSTRACT

The analysis of improved HPLC-UV detector method for the separation and quantification of Acetaminophen, Dextromethorphan hydrobromide and Doxylamine succinate is described. Samples are analysed by means of reverse phase (RP-HPLC) using a Zodiac C-18, (250 × 4.6 mm, 5 µ), and the mobile phase consists of two Channels A and B. Channel-A pH 6.0 Buffer: Methanol (85:15) and Channel-B pH 3.0 Buffer: Acetonitrile (70:30). The flow rate is 1.0 ml/min. The column temperature was maintained at 30˚C and sample temperature was maintained at ambient and wavelength fixed at 245nm UV-detection. It is found that the method of RP-HPLC with UV-detection system for the analysis of Acetaminophen, Dextromethorphan hydrobromide and Doxylamine succinate is straight forward and applied in qualitative and quantitative analysis. The developed LC method was validated with respect to specificity, precision, linearity, ruggedness, stability of analytical solution and robustness. Validation study compared as per ICH guideline.

Keywords: HPLC, Acetaminophen, Dextromethorphan HBr and Doxylamine Succinate, RS

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Received 28 October 2016, Accepted 06 November 2016

Please cite this article as: Rao SVM *et al*., Related Substances of Acetaminophen In Acetaminophen, Dextromethorphan HBr and Doxylamine Succinate Soft Gelatin Capsules by Using RP-HPLC Method. American Journal of PharmTech Research 2016.

INTRODUCTION

Acetaminophen, also known as Paracetamol, is commonly used for its analgesic and antipyretic effects. Its therapeutic effects are similar to salicylates, but it lacks anti-inflammatory, antiplatelet, and gastric ulcerative effects. Dextromethorphan is the d-isomer of the codeine analogue of levorphanol. It shows high affinity binding to several regions of the brain, including the medullary cough Centre. This compound is an NMDA receptor antagonist (receptors-methyl-D-aspartate) and acts as a non-competitive channel blocker. It is one of the widely used antitussives, and is also used to study the involvement of glutamate receptors in neurotoxicity. Doxylamine is a Histamine H1 antagonist with pronounced sedative properties. It is used in allergies and as an antitussive, antiemetic, and hypnotic. Doxylamine has also been administered in veterinary applications and was formerly used in Parkinsonism. It is a combination of a pain reliever, a cough suppressant, and an antihistamine. It is used to treat the aches and pains, cough, fever, headache, runny nose, and sneezing of a cold. This medicine will not treat an infection.

Acetaminophen is chemically known as N-(4-hydroxyphenyl) acetamide is a analgesic determined by UV-spectroscopic and RP-HPLC methods in single and in combined dosage form. Dextromethorphan hydro bromide is chemically known as *Ent*-3-Methoxy-17 methylmorphinan Hydro bromide is a compound an NMDA receptor antagonist and acts as a non-competitive channel blocker. Dextromethorphan hydro bromide in combination with other drugs by using RP-HPLC has been reported. Doxylamine is chemically known as N, N-dimethyl-2-[(1RS)-1-phenyl-1-(pyridin-2-yl-ethoxy-ethanamine.

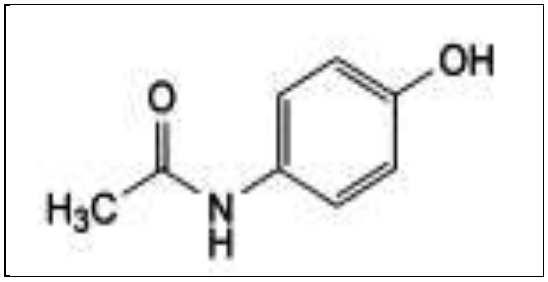

Figure 1: Chemical Structure of Acetaminophen [1]

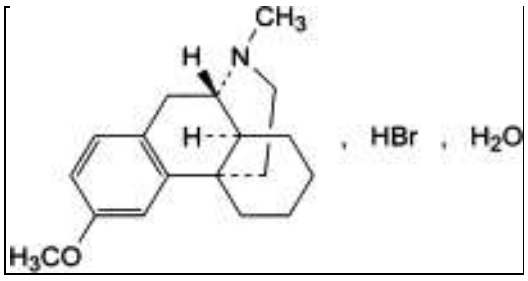

Figure 2: Chemical Structure of Dextromethorphan Hydro Bromide [2]

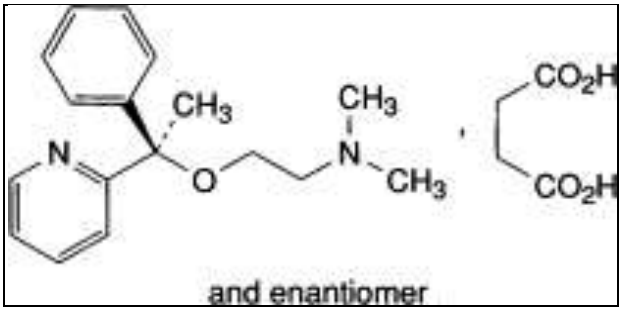

Figure 3: Chemical Structure of Doxylamine Succinate [3]

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and formulations is one of the most challenging tasks. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products. For these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.

In the literature survey, there were several HPLC [4-13] methods that have been reported for the determination of Acetaminophen in pharmaceutical preparation either individually or in combination with other drugs. But no liquid chromatography method has been reported for the simultaneous estimation of Acetaminophen, Dextromethorphan hydrobromide and Doxylamine succinate. Hence, the objective of this study is to develop simple, sensitive and accurate RP-HPLC method for simultaneous estimation of Acetaminophen, Dextromethorphan hydrobromide and Doxylamine succinate in pharmaceutical dosage forms. The developed method was validated according ICH [14-15] guidelines including various stability parameters proved for its accuracy.

Related Substance (impurities) structures:

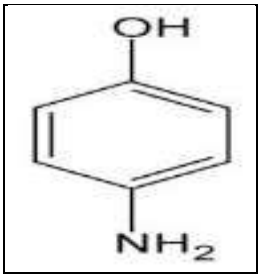

Figure 4: Chemical Structure of 4-Aminophenol [3]

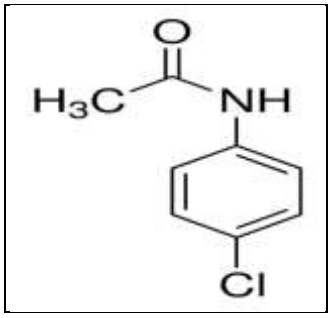

Figure 5: Chemical Structure of 4-chloroacetanilide [3]

MATERIALS AND METHOD

Reagents and chemicals

Pottasium di hydrogen orthophosphate, 1-pentane sulphonic acid sodium salt, Triethyl amine, ortho phosphoric acid, Acetonitrile and Methanol were procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received.

Instrumentation

The analytical separations were carried out on Schimadzu LC-2010 series HPLC system with PDA detector. The analytical column was Zodiac C-18, (250 × 4.6 mm, 5 µ). The mobile phase consists of two Channels A and B. Channel-A pH 6.0 Buffer: Methanol (85:15) and Channel-B pH 3.0 Buffer: Acetonitrile (70:30). The flow rate was 1.0 ml/min. and runtime was 55minutes. Column temperature was maintained at 30˚C and sample temperature was maintained at ambient. Detection was measured 245nm with PDA detector and injection volume was 20µL. The control of HPLC system and data collection was LC solution software.

Preparation of standard and sample solution:

Standard preparation:

Acetaminophen (Solution 1):

50 mg of Acetaminophen working standard was accurately weighed and transferred into a 50 ml volumetric flask, 30ml of diluent was added and sonicated. Then volume was made up with diluent. 5ml of stock solution was pipette out into a 50 ml volumetric flask and finally volume was made up with diluent (100µg/ml).

4-Chloroacetanilide (Solution 2):

20 mg of 4-Chloro acetanilide working standard was accurately weighed and transferred in to a 50 ml volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 5ml of stock solution was pipette out into a 50 ml volumetric flask and volume was made

up with diluent. 5ml of resulting solution was pipette out into a 100ml of volumetric flask and finally volume was made up with diluent (2µg/ml).

4-aminophenol (Solution 3):

50 mg of 4-Aminophenol working standard was accurately weighed and transferred in to a 50 ml volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 10ml of stock solution was pipette out into a 50 ml volumetric flask and finally volume was made up with diluent (200µg/ml).

Resolution solution (Diluted standard):

20 mg of Doxylamine succinate working standard and 10mg of Dextromethorphan HBr working standard were accurately weighed and transferred into a 50ml volumetric flask. 10ml of Solution 1, 5ml of Solution 2, and 5ml of Solution 3 were added into a 50ml volumetric flask and finally volume was made up with diluent.

Sample preparation:

8000 mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transferred into a 100 ml volumetric flask. 70mL of diluent was added and sonicated for 30 minutes. Finally volume was made up with diluent and filtered the solution through whatman filter no: 42.

Spiked sample preparation:

8000 mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transferred into a 100 ml volumetric flask. 10ml of Solution-2, 10ml of Solution-3 and 70mL of diluent were added and sonicated for 30minutes. Finally volume was made up with diluent and filtered the solution through whatman filter no: 42.

RESULTS AND DISCUSSION

Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 245 nm from the absorption spectrum.

Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C_{18} columns could be appropriately used for the separation of related substances for Acetaminophen.

Selection of mobile phase

Different mobile phases and Stationary phases were employed to develop a suitable LC method for quantitative determination of Acetaminophen in its drug. Different mobile phase composition were tried to obtain good peak shape and selectivity for impurities present in Acetaminophen. Resolution was observed when buffer and methanol in the ratio of 70:30 v/v. Resolution of peaks were not satisfactory for 4-Aminophenol, Doxylamine Succinate and Acetaminophen and Dextromethorphan HBr. In the next approach buffer and acetonitrile in a ratio of 70:30 v/v. Resolution of peaks were not satisfactory for 4-Aminophenol, Doxylamine Succinate and Acetaminophen and Dextromethorphan HBr.

In another trial using Zodiac C-18, (250 × 4.6 mm, 5 µ) column with a mobile phase consists of two Channels A and B. Channel-A pH 6.0 Buffer: Methanol (85:15) and Channel-B pH 3.0 Buffer: Acetonitrile (70:30). In this eluent resolution was satisfactory for 4-Aminophenol, Doxylamine Succinate and Acetaminophen and Dextromethorphan HBr gave a very good and well separated from all impurities.

In another trial, solvent system and column are same as above and optimized the column temperature, flow and injection volume. After number of trials for flow and column temperature combination, in order to obtain best column temperature and flow, was set at column temperature 30˚C and flow rate was 1.0 ml/min. runtime 55 minutes with sample temperature maintained at ambient was finally selected. Detection was measured by 245nm with PDA detector and the sample injected was 20µL. These chromatographic conditions were selected for validation studies. The system suitability results obtained using these chromatographic conditions are shown in Table: 1. The standard chromatogram is shown in Figure: 6.

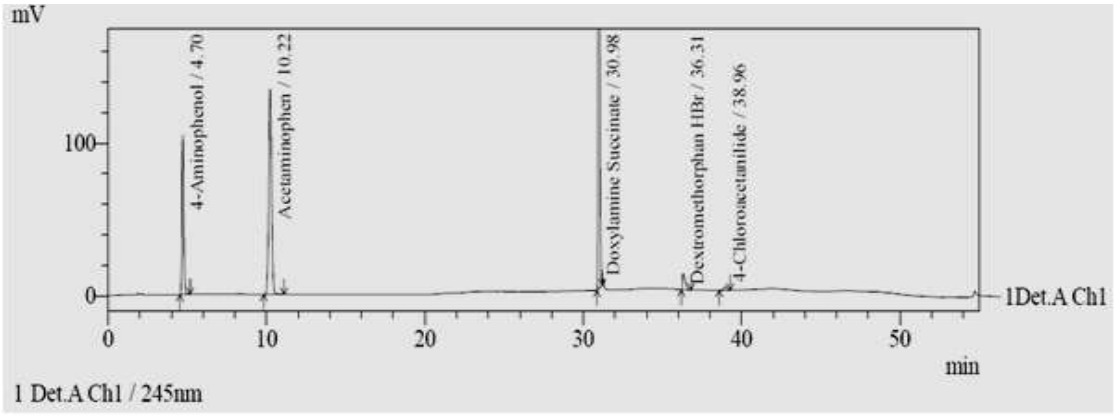

Figure: 6 HPLC Chromatogram for system suitability

Table 1: Summary for system suitability parameters

Name of compound	RT	Theoretical plate	Tailing factor
4-Aminophenol	4.70	8953	1.3
Acetaminophen	10.22	14744	1.1
4-Chloroacetanilide	38.96	207277	1.0

METHOD VALIDATION:

Specificity

Blank interference

A study to establish the interference of blank was conducted. Diluent was injected as per the test method.

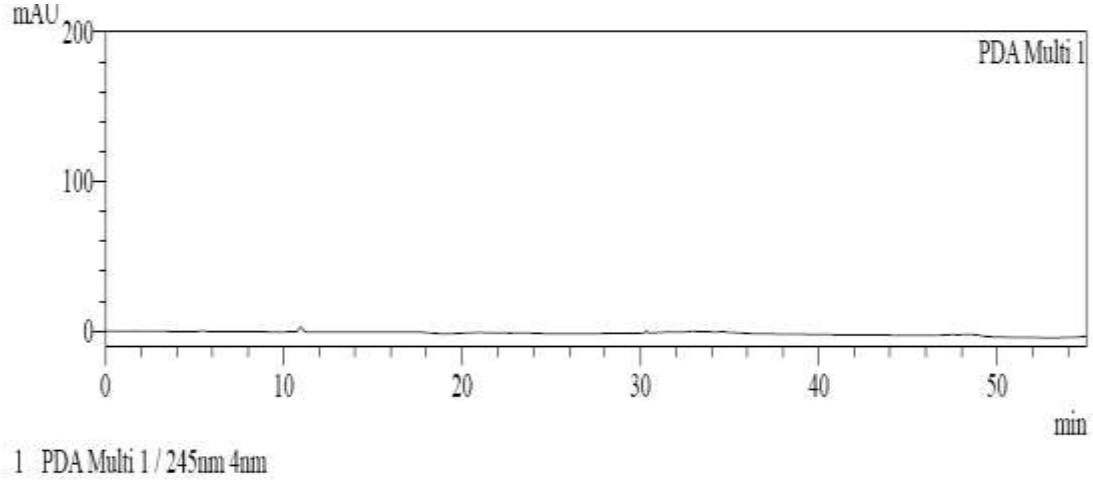

Figure 7: Typical chromatogram of Blank

Placebo interference

A study to establish the interference of placebo was conducted. Sample preparation of placebo was done as that of test sample preparation of assay method. Chromatogram of placebo did not show any additional peaks. This indicated that the excipients used in the formulation did not interfere in

the RS of Acetaminophen, Dextromethorphan HBr and Doxylamine succinate capsules. The details of the impurity interference data was incorporated in the Table:2

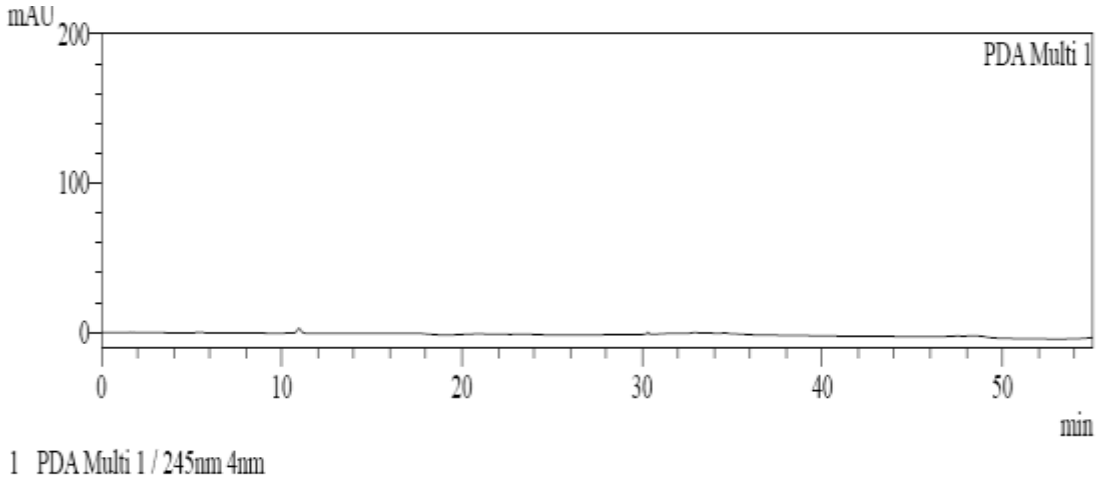

Figure: 8 Typical chromatogram of Placebo

Impurity stock solution preparation for spiked test solution

4-aminophenol

50 mg of 4-Aminophenol working standard was accurately weighed and transferred in to a 50 ml volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 10ml of stock solution was pipette out into a 50 ml volumetric flask and finally volume was made up with diluent. Further diluted 10ml of above solution was pipette out into a 100 ml volumetric flask and finally volume was made up with diluent (20µg/ml).

4-Chloro acetanilide

20 mg of 4-Chloroacetanilide working standard was accurately weighed and transferred in to a 50 ml volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 5ml of stock solution was pipette out into a 50 ml volumetric flask and volume was made up with diluent. 5ml of resulting solution was pipette out into a 100ml of volumetric flask and finally volume was made up with diluent (2µg/ml). Further diluted 10ml of above solution was pipette out into a 100 ml volumetric flask and finally volume was made up with diluent (0.2µg/ml).

Spiked sample preparation

8000 mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transferred into a 100 ml volumetric flask. 10ml of Solution-2, 10ml of Solution-3 and 70mL of diluent were added and sonicated for 30minutes. Finally volume was made up with diluent and filtered the solution through whatman filter no: 42.

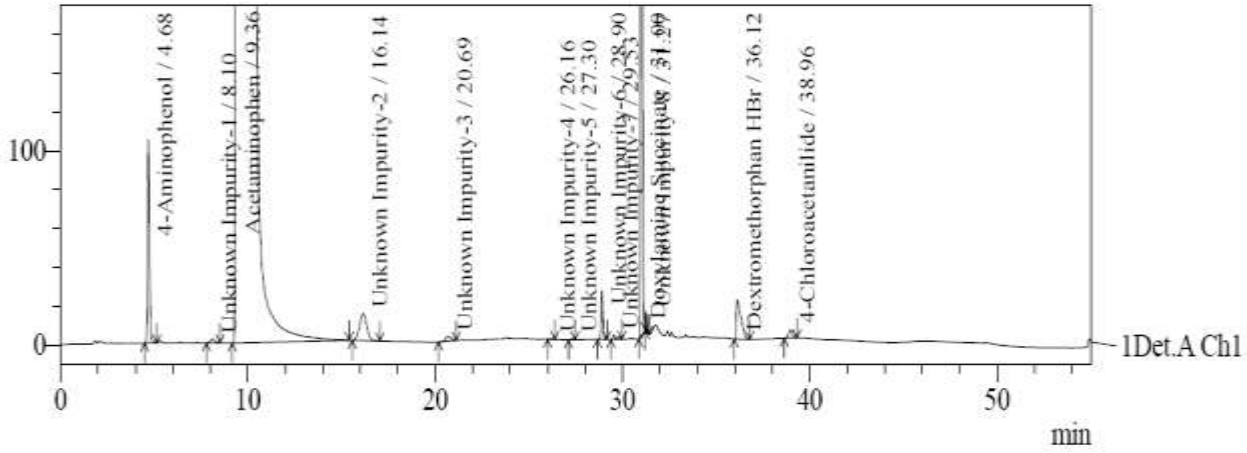

Figure: 9 Typical chromatogram spiked sample

Table 2 Impurity interference data

Preparation	RT	Peak response	Peak Purity index
Blank	NA	NA	NA
Placebo	NA	NA	NA
Standard solution			
4-Aminophenol	4.70	737186	1.000
Acetaminophen	10.22	1691573	1.000
4-Chloroacetanilide	38.96	29664	0.999
Sample with spike			
4-Aminophenol	4.68	736608	1.000
Acetaminophen	9.36	284086155	0.999
4-Chloroacetanilide	38.96	30636	0.999

It was observed that known impurities are not co eluting with each other and main analyte peak. Peak purity of the Acetaminophen in the as such standard solution preparation and in spiked test preparation was calculated and found to be within the acceptable limit.

Method Precision

Precision of the impurities and degradants method was determined by injecting six sample solutions spiked with 4-Aminophenol, 4-Chloroacetanilide at specification level. The samples were prepared as per the method and the result for precision study is tabulated in Table: 3.

Table: 3 Results of method precision

No. of Sample	4-aminophenol	4-Chloroacetanilide	Total Impurity
01	100.2	104.6	0.042
02	100.5	100.7	0.042
03	100.8	102.8	0.043
04	100.0	103.0	0.041
05	101.1	103.6	0.041
06	101.2	104.1	0.042
Mean	100.6	103.1	0.042

SD	0.48	1.33	0.0007
% RSD	**0.5**	**1.3**	**1.6**

The method precession was performed with six replicate solutions of standard solutions prepared and the system suitability parameters found were within the acceptance criteria

4.4 Limit of detection (LOD) & Limit of Quantitation (LOQ).

Table: 4 LOD for Acetaminophen and impurities

Name	Concentration in ppm	% RSD	Signal to noise ratio
4-aminophenol	0.04	9.1	4.1
4-Chloroacetanilide	0.01	6.9	4.9
…	0.02	9.9	3.7

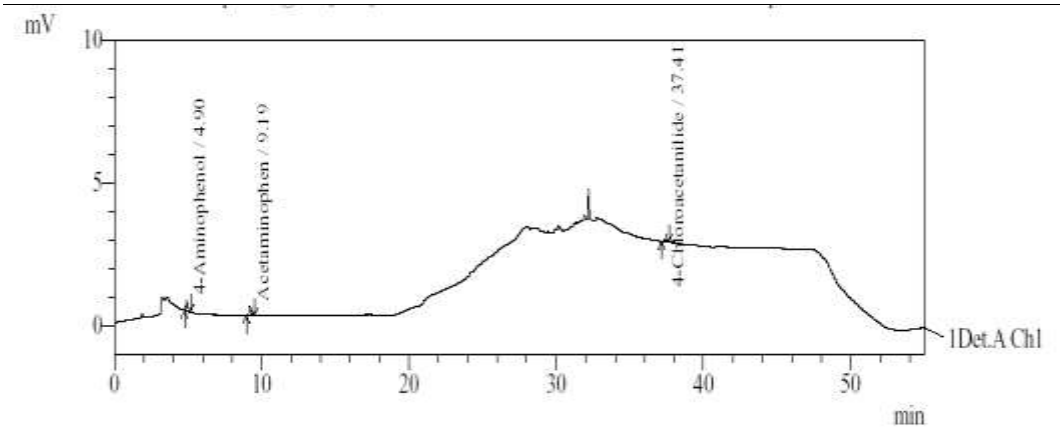

Figure: 10 Chromatogram for LOD

Table: 5 LOQ for Acetaminophen and impurities

Name	Concentration in ppm	% RSD	Signal to noise ratio
4-aminophenol	0.12	5.3	11.5
4-Chloroacetanilide	0.03	2.9	10.6
Acetaminophen	0.06	1.4	10.5

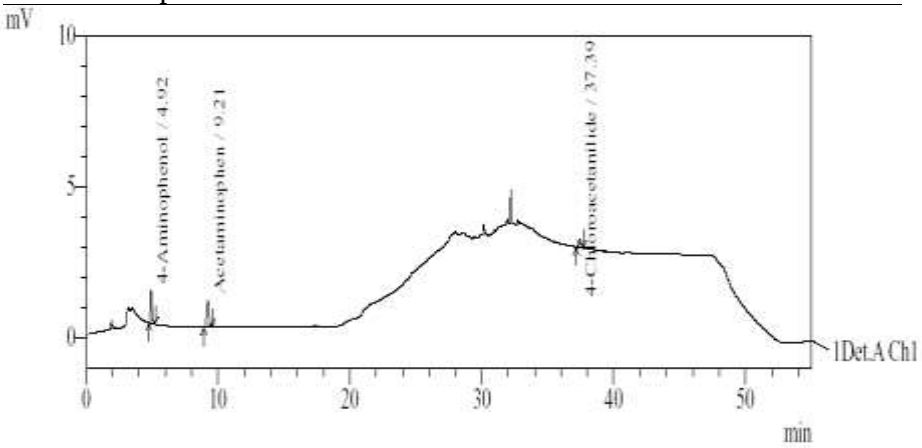

Figure: 11 Chromatogram for LOQ

The limit of detection and limit of quantitation values obtained for each impurity and Acetaminophen are within the acceptance criteria. **Table 4 and Table: 5**

Linearity and Range

Standard solutions of Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide in the concentration levels from 50 % to 150 % of standard solution were injected into HPLC system. The linearity graph was plotted from 50 % to 150% of drug concentration. Report the linearity range as the range for determining the impurities. Results obtained are in the tables (Table 6 and Table 7) & figures show the line of best fit for peak area versus concentration for each impurity.

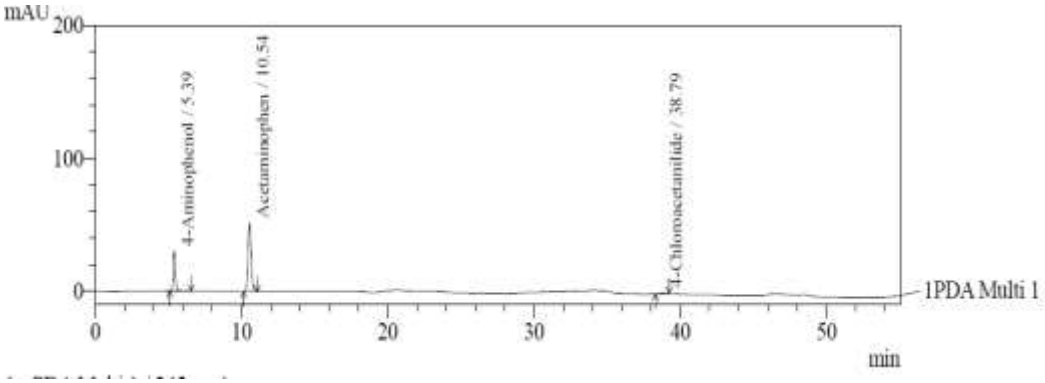

Figure: 12 Chromatogram for linearity-50%

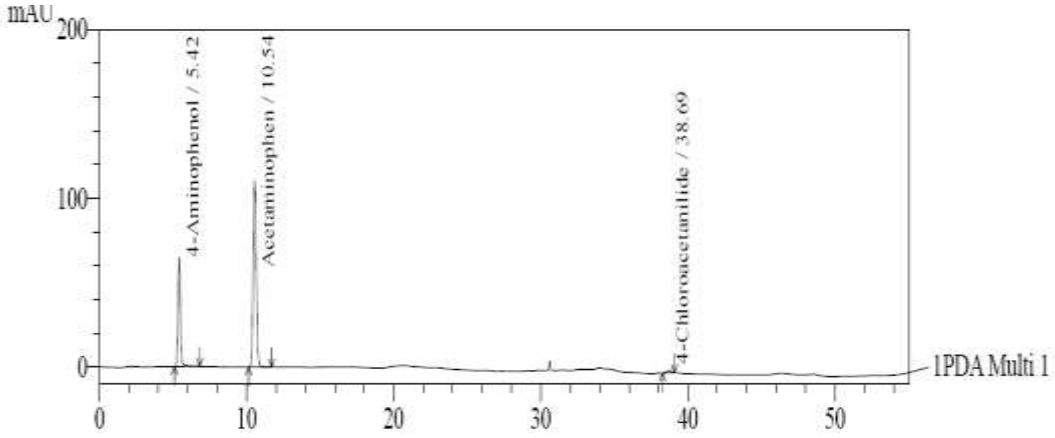

Figure: 13 Chromatogram for linearity-100%

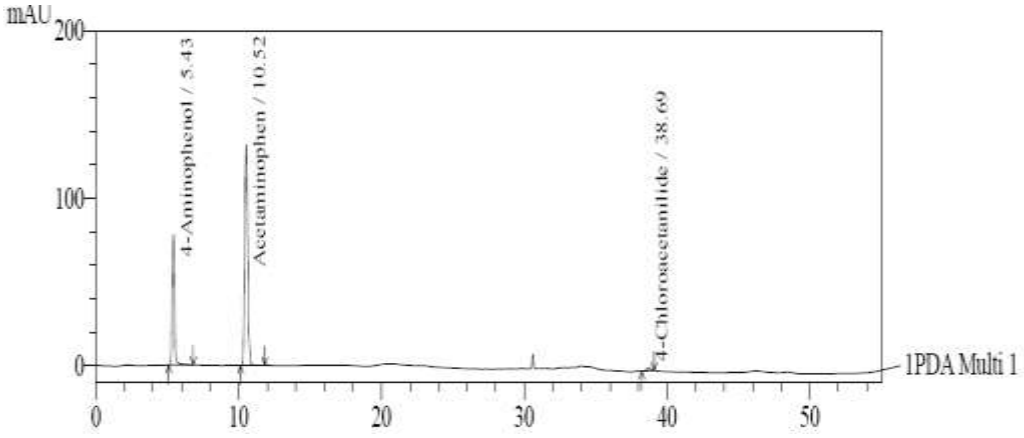

Figure: 14 Chromatogram for linearity-125%

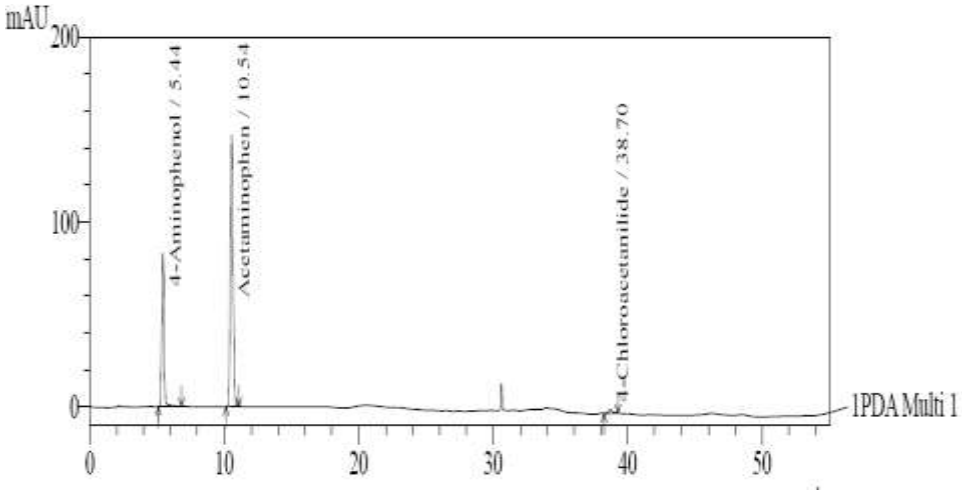

Figure: 15 Chromatogram for linearity-150%

Table: 6 Linearity of Acetaminophen and impurities

Sample No.	% level	Peak area		
		Acetaminophen	4-Aminophenol	4-Chloroacetanilide
1	LOQ	5587	4533	3853
2	50	827582	335067	15170
3	75	1257022	534750	22429
4	100	1659744	726694	30540
5	125	2024201	889101	38574
6	150	2446583	1092212	45504

Table: 7 Regression analyses for the linearity graph

Linear regression analysis	Acetaminophen	4-Aminophenol	4-Chloroacetanilide
Correlation coefficient (r^2)	0.999	0.998	0.999
Y- intercept	-2389	3612	-100.6
Slope	18660	6586	305.6

Accuracy

Recovery of Acetaminophen impurities in Acetaminophen was performed. The sample was taken and varying amounts of Acetaminophen impurities representing LOQ to 150 % of specification level were added to the flasks. The spiked samples were prepared as per the method and the results are tabulated in **Table 8.**

Table: 8 Accuracy study of Acetaminophen

S.No.	Theoretical (%)	% Mean Recovery ± %RSD	
		4-Aminophenol	4-Chloroacetanilide
1	LOQ	99.7±1.4	100.3±1.5
2	50	96.6±1.7	96.1±1.7
3	100	99.6±0.4	98.1±1.1
4	150	100.6±0.4	102.4± 1.3

RESULTS AND DISCUSSION:

A simple, economic, accurate and precise HPLC method was successfully developed. In this method it was carried out by using Zodiac C18 (250× 4.6mm) with 5µm particle size. Injection volume of 20µl is injected and eluted with the mobile phase consists of two Channels A and B. Channel-A pH 6.0 Buffer: Methanol (85:15) and Channel-B pH 3.0 Buffer: Acetonitrile (70:30)., which is pumped at a flow rate of 1.0 ml/min with column and sampler temperatures at 30˚C and ambient respectively and runtime was optimized to 55 min. Detection was carried out at 245 nm. The peaks obtained were sharp with retention time of 4.70 for 4-Aminophenol, 38.96 for 4-Chloroacetanilide, 10.22 for Acetaminophen, 30.98 for Doxylamine succinate and 36.31 for Dextromethorphan HBr. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness, and stability of solution. For Selectivity, the chromatograms were recorded for standard and sample solutions of Acetaminophen and its related substances. Selectivity studies reveal that the peak is well separated from each other.

The limit of detection (LOD) and limit of quantitation (LOQ) for 4-Aminophenol was found to be 0.04µg/ml,0.12µg/ml, for 4-Chloroacetanilide 0.01µg/ml,0.03µg/ml and Acetaminophen 0.02µg/ml,0.06µg/ml, respectively. The linearity results for Acetaminophen and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Acetaminophen and its impurities found to be 0.999, 0.998 and 0.999 respectively. The accuracy studies were shown as % recovery for Acetaminophen and its impurities at LOQ, 50%, 100% and 150%.The limit of % recovered shown is in the range of 90 and 110% and the results obtained were found to be within

the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the impurities in the range 96.6-102.4 respectively. For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Acetaminophen and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits.

CONCLUSION

The author has developed a novel chromatographic method developed for the assay the related substances of acetaminophen in acetaminophen, Doxylamine Succinate and Dextromethorphan HBr in soft gelatin capsules. From the results, it was observed that the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose. Hence it was concluded that this method could be used for the routine estimation of related substances of acetaminophen in acetaminophen, Doxylamine Succinate Dextromethorphan HBr in soft gelatin capsules.

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