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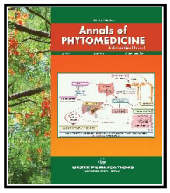

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Analytical method validation for related substance of acetaminophen soft gelatin capsules by HPLC chromatography study

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Abstract

Acetaminophen is the name most usually used in the United States and Japan, but paracetamol is more commonly used in other parts of the world, including Europe, Australia, and Asia, which is commonly used as a pain reliever and fever reducer. The current work sought to create an analytical method for the related substance of acetaminophen in soft gelatin capsules. The presence of related compounds in acetaminophen formulations can affect their safety and efficacy. In this study, a high-performance liquid chromatography (HPLC) approach for detecting related compounds in acetaminophen was devised.The procedure involved solvent extraction of the material, followed by chromatographic separation on a C8 column with a mobile phase of 250 ml of methanol, 1.15 g of 40% w/v solution of tetra butyl ammonium hydroxide, 375 ml of 0.05 M disodium hydrogen orthophosphate and 375 ml of 0.05 M sodium dihydrogen orthophosphate. A PDA detector set to the appropriate wavelength of 245 nm was used for detection. The run time of solutions 2 and 3 was 10 min, and solutions 1, 4, blank, placebo, and spiked sample solutions were 50 min. The developed approach was validated using ICH criteria for factors such as specificity, accuracy, precision, and stability. The approach showed high specificity, with resolution between the acetaminophen peak and related compounds. The new HPLC method was successfully used to analyze genuine acetaminophen samples, indicating its suitability for routine quality control in pharmaceutical laboratories. This method provides a dependable means for determining related chemicals in acetaminophen, assuring the safety and efficacy of pharmaceutical products containing this widely used medication.

1. Introduction

Acetaminophen is a popular over-the-counter medication that is well renowned for its ability to reduce fever and relieve pain. It is also referred to as paracetamol in many countries. The effectiveness and safety of acetaminophen are dependent on the quality and purity of its formulations, despite its widespread use (Abdulbari and Ihsan, 2013). The creation of an analytical technique for evaluating related chemicals in formulations containing acetaminophen is described in this abstract. High-performance liquid chromatography (HPLC), a tried-and-true method for pharmaceutical analysis noted for its sensitivity and specificity, is used in this process (Afkhami *et al.,* 2014; Varuni *et al.,* 2023). The approach produces good resolution between acetaminophen and associated contaminants by carefully optimizing chromatographic parameters, such as column selection, mobile phase composition, and detection wavelength (Akella Anuradha *et al.,* 2023). The robustness of the approach is validated by validation studies, which show acceptable specificity, linearity, accuracy, and precision (Birajdar *et al.,* 2009; Gadhimathi *et al.,* 2023). The method that has been developed provides a dependable way to measure relevant compounds in formulations containing acetaminophen, guaranteeing adherence to regulatory requirements and improving pharmaceutical quality control procedures (Craig and Stitzel, 2004).

Due to its adaptability, sensibility, and ability to discriminate complicated mixtures, HPLC is one of the methods frequently employed for the analysis of acetaminophen (Gharge and Dhabale, 2010). The literature has published a wide range of HPLC procedures that use various mobile phases, stationary phases, and detection methods such as mass spectrometry (MS), diode array detection (DAD), and UV-visible spectroscopy (Gowramma *et al.,* 2010; Nivetha *et al.,* 2023). The literature review highlights the wide variety of analytical techniques that are available for determining the presence of acetaminophen in biological samples and pharmaceutical formulations (Indumathy *et al.,* 2023). The choice of a suitable methodology is contingent upon various aspects, including sample matrix complexity, instrumentation availability, and sensitivity requirements (Graham *et al.,* 2013). Each method has its own set of benefits and drawbacks. To solve new issues and enhance the accuracy and effectiveness of acetaminophen analysis in diverse settings, ongoing research and method improvement are crucial (Mahaparale *et al.,* 2007).

2. Materials and Methods

2.1 Chemicals and reagents

Acetaminophen capsules contain acetaminophen 500 mg of methanol (HPLC grade), 40% of tetra butyl ammonium hydroxide, disodium

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hydrogen orthophosphate, and sodium dihydrogen orthophosphate. Glassware that had been calibrated was used for the entire endeavor. In this investigation, every reagent utilized was AR grade.

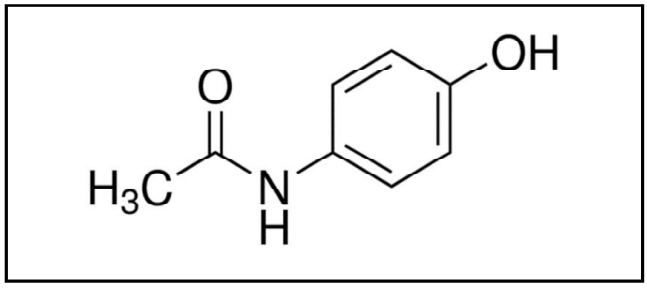

Figure 1: Structure of acetaminophen.

2.2 Instrument details

HPLC with PDA detector was used to detect the compound wavelength, microbalance for measurement of the minimum volume of substances, analytical balance for the measurement of samples and other substances, ultrasonicator was used for agitating the particles in liquids, ultrasonicator bath was used for degassing the liquids, and the stationary phase 250 mm × 4.6 mm × 5 µm, C8 column was used in the study.

2.3 Preparation of solutions

2.3.1 Mobile phase

250 ml of methanol, 1.15 g of 40% of tetra butyl ammonium hydroxide, 375 ml of 0.05 M disodium hydrogen orthophosphate, and 375 ml of 0.05 M of sodium dihydrogen orthophosphate (Monica *et al.,* 2003).

2.3.2 0.05 M disodium hydrogen orthophosphate

Weigh and dissolve 7.1 g of disodium hydrogen orthophosphate in 1000 ml of water.

2.3.3 0.05 M sodium dihydrogen orthophosphate

Weigh and dissolve 6.0 g of sodium dihydrogen orthophosphate in 1000 ml of water.

2.3.4 Diluent - Mobile phase

2.3.5 Blank - Diluent

2.3.6 Solution 1 (Conc. 20000 ppm of acetaminophen)

Weighed 20 capsules and sliced the capsules open. Gather the medication into a sterile petri dish. Determine the average fill weight by weighing the empty capsule. Precisely weigh out approximately 400 mg of medication (which is equal to 200 mg of acetaminophen) in a 10 ml volumetric flask. Next, add 8 ml of diluent, sonicate for five minutes, dilute with diluent to volume, and thoroughly mix. Pass the mixture through a nylon syringe filter with a 0.45 µm pore size.

2.3.7 Solution 2 (Conc. 50 ppm of acetaminophen)

Use diluent to dilute 1 ml of solution 1 to 20 ml, then thoroughly mix. Using diluent, further dilute 1 ml of this solution to 20 ml and thoroughly mix.

2.3.8 Solution 3 (Conc. 20 ppm [0.002%] each of 4-aminophenol and acetaminophen)

Each 20 mg of 4-aminophenol and acetaminophen should be precisely weighed. Then, pour the mixture into a 100 mlvolumetric flask, add 70 ml of diluent, and sonicate to dissolve. Next, add diluent to make up the volume and thoroughly mix. Using diluent, further dilute the afore mentioned solution by 10 ml to 100 ml.

2.3.9 Solution 4 (Conc. 0.2 ppm [0.00002%] of 4-chloroacetanilide)

Accurately weigh out 20 mg of 4-chloroacetanilide in a 100 ml volumetric flask. Then, add 5 ml of methanol, sonicate to dissolve the mixture, and add additional methanol to make up the volume. Mix thoroughly after dilution (1 ml of the aforementioned solution to 100 ml). Using diluent, further dilute 1 ml of the afore mentioned solution to 10 ml and thoroughly mix.

2.3.10 Placebo solution

Cut twenty placebo capsules. Gather the medication into a sterile Petri dish. Precisely weigh out 200 mg of medication into a 10 ml volumetric flask, then add 8 ml of diluent. Sonicate for five min, then dilute with diluent to volume and thoroughly mix. Pass the mixture through a nylon syringe filter with a 0.45 µm pore size (Shihana *et al.,* 2010).

2.3.11 Chromatographic conditions

HPLC with PDA detector, the stationary phase 250 mm × 4.6 mm × 5 µm C_8 column was used (Subhamalar *et al.,* 2023). The sample flows at the rate of 1.5 ml per minute. 35°C of column oven temperature was maintained, the volume of the injection was 20 µl, and the detector (PDA) detected the compound at the wavelength of 245 nm. The run time of solution 2 and solution 3 was 10 min, and solution 1, solution 4, blank, placebo, and spiked sample solution were 50 min (Vaidya *et al.,* 2010).

3. Results

3.1 System suitability

Acetaminophen standard and system suitability solutions are made and injected into the HPLC system. The mean value of RT is 2.6 min and area of 492813 for 4-aminophenol, and RT is 40.9 min and area of 13931 for 4-chloroacetanilide and RT is 3.9 min and an area of 2682632 for acetaminophen.

3.2 Specificity

At the 4-aminophenol, 4-chloroacetanilide, and acetaminophen retention times, the results indicate that there is no interference from blank and placebo peaks. The acceptance criteria are met by the peak purity index values of acetaminophen, 4-aminophenol, and 4-chloroacetanilide in both the standard and sample.

3.3 Establishment of LOD and LOQ

3.3.1 Establishment of LOD

The LOD level concentration of 4-aminophenol and 4-chloroacetanilide was established and observed. The mean value of the LOD level concentration of 4-aminophenol is RT 2.6 min and area 538822, the LOD level of concentration of 4-chloroacetanilide is RT 38.4 min and area 12059, the LOD level of concentration of acetaminophen is RT 3.8 min and area 3627353.

Table 1: Specificity report of acetaminophen

System suitability parameters	Observation					Acceptance criteria
	Blank	Placebo	Standard	Unspiked sample	Spiked sample	
Interference	NI	NI	NA	NA	NA	There should not be any interference by blank, placebo, or any impurity peaks at the RT of the main peak and also there should not be any interference of known impurities at the RT of each other.
RT of acetaminophen	NA	NA	3.9	4.0	4.0	
RT of 4-aminophenol	NA	NA	2.6	ND	2.6	
RT of 4-chloroacetanilide	NA	NA	40.0	ND	39.8	
Area of acetaminophen	NA	NA	2834318	90143011	90029400	NA
Area of 4-aminophenol	NA	NA	442662	ND	473692	
Area of 4-chloroacetanilide	NA	NA	12086	ND	12510	
Peak purity index of	NA	NA	1.000	0.999	0.999	The peak purity index of the principal acetaminophen peak and known impurities peak from blank, placebo, standard, unspiked sample, and spiked sample preparation should be not less than 0.99
Peak purity index of 4-aminophenol	NA	NA	1.000	ND	1.000	
Peak purityiIndex of 4-chloroacetanilide	NA	NA	0.999	ND	1.000	

NI - Not interference; NA - Not applicable; ND - Not detected

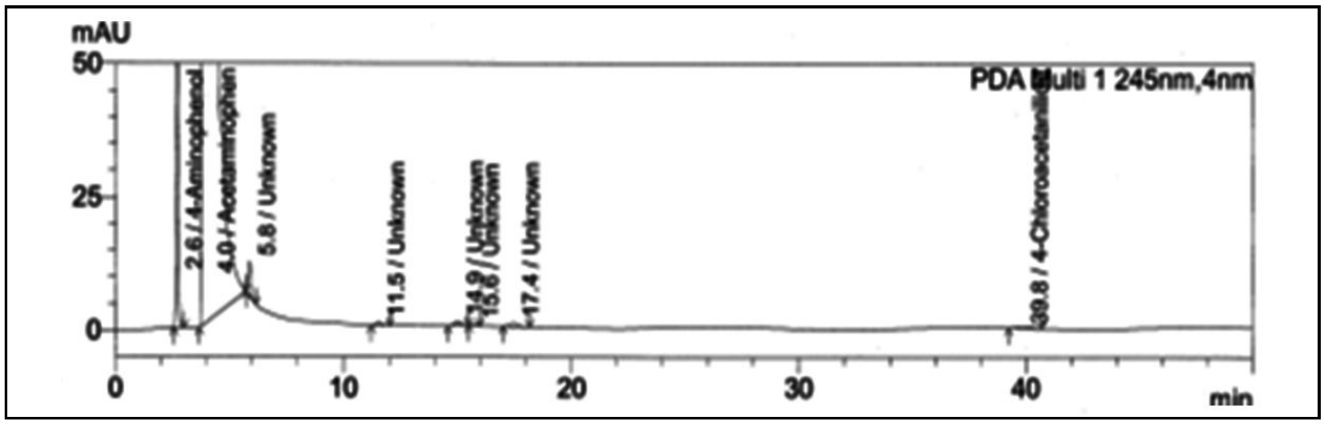

Figure 2: Spiked sample of acetaminophen.

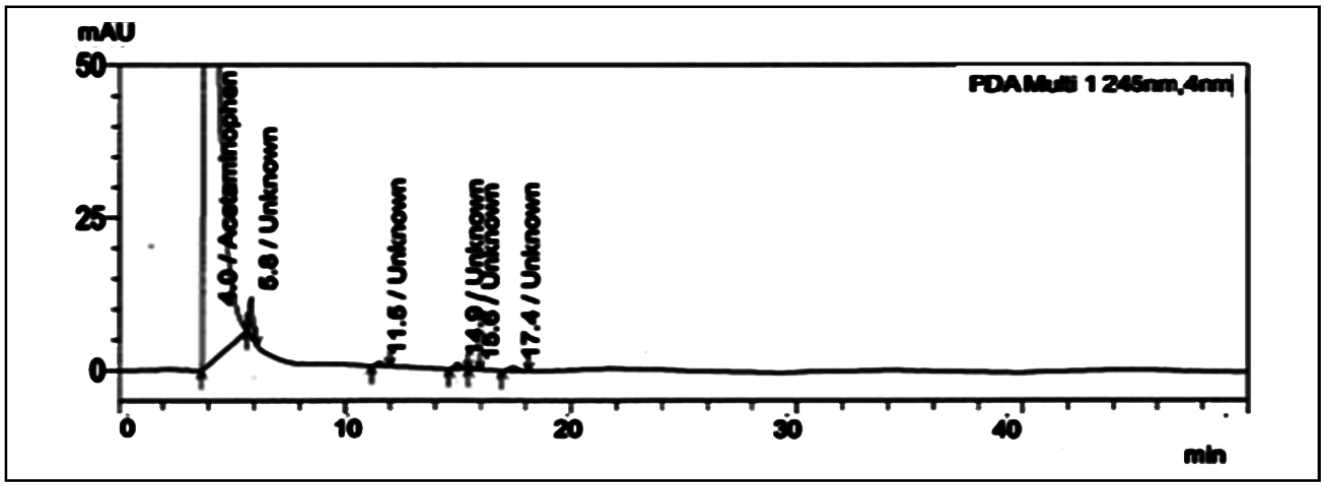

Figure 3: Unspiked sample of acetaminophen.

The established LOD level concentration is 4-aminophenol LOD solution-2, the observed LOD value found to be 0.0502 ppm concerning signal-to-noise ratio is 16.7 S/N ratio, 4-chloroacetanilide LOD solution 2, the observed LOD value found to be 0.0293 ppm with respect to signal-to-noise ratio is 3.7 S/N ratio for established LOD concentration found to be within the acceptable limit of 3. From the established level, 3 times of LOD concentration is considered as LOQ.

3.3.2 Establishment of LOD

The LOD level of acetaminophen was established and observed. The mean value of the LOD level concentration of 4-aminophenol is RT 2.6 min and area 544427, the LOD level of concentration of 4-chloroacetanilide is RT 36.1 min and area 14979, the LOD level of concentration of acetaminophen is RT 3.8 min and area 2926398.

The established LOD level concentration is acetaminophen LOD solution 2, the observed LOD value found to be 0.0149 ppm with respect to to noise ratio is 8.5 S/N ratio for established LOD concentration found to be within the acceptable limit of 3. From the established level, 3 times of LOD concentration is considered as LOQ.

3.3.3 Establishment of LOQ

3 times of established LOD concentration shall be considered as LOQ.

3.3.4 Precision at LOQ

The precision at the LOQ level of 4-aminophenol, 4-chloroacetanilide, and acetaminophen was established and observed. The mean value of the LOD level concentration of 4-aminophenol is RT 2.6 and area 529548 and the standard deviation is RT 0.0 and area 89.8 and the %RSD is RT 0.0 and area 0.0, the LOD level of concentration of 4-chloroacetanilide is RT 35.7 and area 12667 and the standard deviation is RT 0.0 and area 150.6 and the %RSD is RT 0.0 and area 1.1, the LOD level of concentration of acetaminophen is RT 3.7 and area 2926637 and the standard deviation is RT 0.0 and area 2883.4 and the % RSD is RT 0.0 and area 0.1.

The established LOQ value of 4-chloroacetanilide with respect to concentration is 0.0905 ppm and signal-to-noise ratio is found to be 12.9. The established LOQ value of 4-aminophenol with respect to concentration is 0.1523 ppm and signal-to-noise ratio is found to be 66.9.The established LOQ value of acetaminophen with respect to concentration is 0.0452 ppm and signal-to-noise ratio is found to be 46.3.

3.4 Accuracy

The degree to which test results produced by the method closely resemble the genuine value is known as accuracy. The percentage recovery by the content of known, introduced amounts of analytes is a common way to express accuracy. A measure of an analytical method's exactness is its accuracy. The 4 concentrations (LOQ, 50%, 100%, and 150%) were used to evaluate accuracy. Solutions for standard and spiked samples are made at LOQ, 50%, 100%, and 150% concentrations. The area acquired for every concentration is used to compute the percentage of recovery.

Table 2: Accuracy report of acetaminophen

Solution	Concentration	%Recovery	%RSD
4-aminophenol	LOQ	126.9	1.6
	50%	85.7	1.2
	100%	82.4	1.3
	150%	82.1	1.7
4-chloroacetanilide	LOQ	90.9	1.1
	50%	108.2	1.7
	100%	94.4	1.3
	150%	98.5	1.5
Acetaminophen	LOQ	113.8	1.8
	50%	95.7	0.0
	100%	102.3	1.8
	150%	97.4	0.1

The acceptance requirements were met by the average percentage recovery at each concentration level (LOQ, 50%, 100%, and 150%) and the percentage RSD for recovery. As a result, it can be said that the approach is accurate and exact between LOQ and 150% of the specification limit while taking sample concentration into account.

3.5 Precision

3.5.1 System precision

Six replicates of a standard acetaminophen solution were prepared and injected into the HPLC system. The mean value of the system precision of 4-aminophenol is RT 2.6 min and area 492813, the system precision of 4-chloroacetanilide is RT 40.9 min and area 13931, the system precision of acetaminophen is RT 3.9 min and area 2682632.

The acceptance requirements are met by the system suitability characteristics. Because of this, the system is appropriate for estimating associated compounds of acetaminophen in 500 mg capsules of acetaminophen.

3.5.2 Method precision

By examining the 500 mg of acetaminophen capsules in six replicate spiking sample preparations, the method precision of the solution will be shown. The mean value of the method precision of 4-aminophenol is RT 2.6 min and area 547627, the method precision of

4-chloroacetanilide is RT 41.5 min and area 13080, the method precision of acetaminophen is RT 3.9 min and area 4211224.

The unspiked sample preparations are demonstrated in method precision by analyzing the acetaminophen capsules 500 mg in 6 replicates. The mean value of the method precision of 4-aminophenol, the %recovery is 99.9, the standard deviation is 1.7281 and the %RSD is 1.7, and 4-chloroacetanilide, the %recovery is 100.0, the standard deviation is 0.0 and the %RSD is 0.0.

Six replicate spiked preparations yielded 4-aminophenol and 4-chloroacetanilide findings, and the % RSD values obtained fall within the acceptable range. The %RSD values obtained and the 4-aminophenol and 4-chloroacetanilide results of two replicated, unspiked preparations fall within the acceptable range. This demonstrates the method is precise and repeatable.

3.5.3 Intermediate precision

Analyzing the same batch of related substance in 500 mg acetaminophen capsules as in precision with six replicate samples, in a separate lab with a different analyst, using a different instrument and column on a different day, demonstrates intermediate precision.

Table 3: Intermediate precision report of acetaminophen

Sample No.	4-aminophenol		4-chloroacetanilide	
	MP	IMP	MP	IMP
1	101.6230	111.5841	100.0000	100.0000
2	100.1050	109.1690	100.0000	90.0000
3	101.6840	114.5688	100.0000	100.0000
4	98.3572	112.3181	100.0000	90.0000
5	100.4150	109.7963	100.0000	90.0000
6	97.4279	110.6589	100.0000	90.0000
Average	99.9353	111.3492	100.0000	93.3333
Average of 12 units	105.6	96.7		
Standard deviation	1.7287	1.9494	0.0	5.1639
The standard deviation of 12 units	6.2141	4.9236		
RSD	1.7	1.7	0.0	5.5
RSD of 12 units	5.8	5.0		

MP - Method precision; IMP - Intermediate precision

Six replicate spiking preparations yielded findings for 4-aminophenol and 4-chloroacetanilide by analyst I and analyst II, and the percentage RSD values obtained fall within the acceptable range. The findings of the two replicate unspiked preparations 4-aminophenol and 4-chloroacetanilide analyses, as well as the percentage RSD values obtained by analyst I and analyst II using two distinct HPLC systems, column, and two distinct days of work, fall within the acceptable range. This indicates that the procedure is accurate and repeatable.

3.6 Solution stability

By injecting standard and sample solutions for up to 48 h, the stability of the solution is shown. The mean value of the intermediate precision of 4-aminophenol is RT 2.6 min and area 492813, the intermediate precision of 4-chloroacetanilide is RT 40.9 min and area 13931, the intermediate precision of acetaminophen is RT 3.9 min and area 2682632.

Both the standard and sample solutions are stable for up to 24 h, as indicated by the %RSD of the area of standard and sample solution-02 acquired at different time intervals. Standard and sample solution-03's %RSD of the area acquired with varying time intervals demonstrates that the former is stable for up to 16 h. and the latter for up to 24 h. Both the standard and sample solutions are stable for up to 12 h, as indicated by the %RSD of the area of the standard and sample solution-02 acquired at different time intervals.

4. Discussion

For the quantitative determination of acetaminophen, high-performance liquid chromatography (HPLC) has shown to be a useful analytical method, especially when used in conjunction with relative substance analysis. Because of its limited treatment window and potential side effects, accurate measurement of acetaminophen is crucial. The thorough investigation of numerous chromatographic parameters, sample preparation methods, and validation factors highlights how crucial method development and validation are to guarantee precise and trustworthy findings. To obtain enough resolution and sensitivity for acetaminophen peaks within complicated matrices, chromatographic settings must be optimized. This includes choosing the right columns, the makeup of the mobile phase, and the detection wavelength. Additionally, isolating acetaminophen from interfering substances.

5. Conclusion

The method for the estimation of associated drug acetaminophen in the "Method verification report for acetaminophen in acetaminophen capsules 500 mg" by HPLC method is appropriate, exact, accurate, specific, and stable, according to the results of the method validation. As a result, this approach is deemed validated and suitable for ongoing investigation.

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Conflict of interest

The authors declare no conflict of interest relevant to this article.

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