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Development and Validation of RP-HPLC Method for Simultaneous Estimation of Amoxicillin trihydrate and Flucloxacillin sodium in capsule dosage form

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Abstract: A reverse phase high performance liquid chromatographic method was developed for simultaneous determination of amoxicillin trihydrate and flucloxacillin sodium in bulk and pharmaceutical formulation. The separation was made by a Kromasil C_{18} column (250 cm × 4.6 mm, 5µm) using 0.020 M potassium dihydrogen orthophosphate - acetonitrile (75:25) as mobile phase. The validation of the method was performed, and specificity, reproducibility, precision and accuracy were confirmed. The limits of quantification were approximately 0.16 µg/ml for amoxicillin trihydrate and 0.25 µg/ml for flucloxacillin sodium. Due to simplicity and accuracy the method particularly suitable for routine pharmaceutical quality control.
Key Words: RP-HPLC, Amoxicillin trihydrate, Flucloxacillin sodium

1. Introduction

Amoxicillin trihydrate (AMT) is chemically a (2S, 5R, 6R) [[(2R)- 2 - amino-2 (4 hydoxyphenyl) acetyl] amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptanes-2-carboxyic acid, and it belongs to class of antibiotics.[1] Amoxicillin trihydrate is official in BP and Eur pharmacopoeia.[2,3] Flucloxacillin sodium (FLU) is chemically (2S,5R,6R)-6-[[[3-(2-chloro-6-flurophenyl)-5-methoxylisoxazol-4-yl] carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptanes-2-carboxylate and it belongs to class of antibiotics having bactericidal action.[1] Flucloxacillin sodium is official in BP and Eur Pharmacopoeia.[2,3] In literature spectrophotometric,[4,5] few HPLC,[6-9] LC-MS,[10] Electospray mass spectroscopy[11] and DSC[12] have been reported for determination of AMT alone and combination with other drugs for pharmaceutical formulation and biological fluids. Spectrophotometric,[13] and HPLC[14,15] methods have been reported for determination of FLU alone and for combination of AMT and FLU chemometric-assisted spectrophotometry,[16] second derivative ratio spectrophotometry and LC,[17] methods for binary mixtures have been reported. But no method developed for combination of AMT and FLU for their simultaneous determination for pharmaceutical formulation. A successful attempt has been made for simultaneous determination of AMT and FLU in combined dosage form. Therefore, it was thought worthwhile to developed simple, precise, accurate and reliable RP-HPLC method for simultaneous estimation of both the dug in combined dosage form.

2. Experimental

2.1. Standards and reagents

Amoxicillin trihydrate (AMT) and Flucloxacillin sodium (FLU) was provided by Concept Pharmaceuticals Ltd. (Aurangabad, India) , was used as

a working standard. The commercially available capsule formulation. Flumox® Capsules was used for quantitative determination. Potassium dihydrogen orthophosphate was of analytical grade. Acetonitrile was HPLC grade purchased from Merck. Chem. Ltd., Mumbai. All solutions were prepared with double distilled R.O water for HPLC.

2.2. Instrumentation

The HPLC system was model Shimadzu LC-2010 composing quaternary pump, autosampler, mobile phase degaser, heated column thermostat, and variable UV detector. The mobile phase contained 0.020 M potassium dihydrogen orthophosphate – acetonitrile (75:25) and flow rate was maintained at 1.5 ml/min and monitored at 225 nm. Chromatographic separations were performed at ambient temperature on a Kromasil C_{18} column (250 cm × 4.6 mm, 5µm), and the injection volume was 20 µl.

2.3. Standard stock solution

About 50 mg of each reference standard AMT and FLU was weighed accurately and transferred to 50 ml volumetric flask. Both drugs were dissolved in 25 ml mobile phase shaking and volume was made up to the mark. further pipette out 5 ml of this solution transferred in to 50 ml volumetric flask and diluted up to 50 ml with mobile phase to get the concentration 100 µg/ml of AMT and FLU. This stock solution was filtered with 0.45 µm (Millifilter, Milford, MA) filter paper.

2.4. Calibration curves

From stock solution the different concentrations were prepared with appropriate dilutions in the range of 2.5 – 20 µg/ml for AMT and FLU. All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area *vs* the drug concentration. The areas exhibited linear responses with $r^2 = 0.9994$ for AMT and $r^2=0.9995$ for FLU. The results are shown in Table 1.

2.5. Analysis of bulk sample

Accurately weighed 50 mg of AMT and 50 mg of FLU were transferred to 50 ml volumetric flask, dissolved in mobile phase and volume was adjusted to mark. Solution was further diluted to get concentration of 10 µg/ml of AMT and 10 µg/ml of FLU was subjected to proposed method, injected with application volume 20 µl and amount of AMT and FLU was determined. The procedure was repeated for six times. The typical Chromatogram is shown in Fig. 1 and results are shown in Table 2.

2.6. Analysis of capsule formulation

To determine the content of AMT and FLU in capsules (Label claim 250 mg AMT and FLU capsule); the twenty capsules were weighed, their mean weight determined. and powder equivalent 50 mg was transferred into a 50 ml volumetric flask containing 25 ml mobile phase, sonicated for 5 min. and diluted to 50 ml with mobile phase. The resulting solution was filtered, using 0.45 µm filter (Millifilter, Milford, MA). The solution was further diluted to get concentration 10 µg/ml AMT an FLU was subjected to proposed method, injected with application volume 20 µl, and amount of AMT and FLU was determined. The assay procedure was repeated for five times; chromatogram of capsule solution is shown in Fig. 2 and results are shown in Table 3.

3. Validation of HPLC method

3.1. Specificity and Selectivity

The specificity of the RP-HPLC method was determined by comparison of the chromatogram of mixed standards and sample solutions. The parameters like retention time (*t*R), resolution (*R*S) and tailing factor (*T*f) were calculated. Good correlation was found between the results of mixed standards and sample solution. The method is quite selective. showed no interfering peak around the retention time of AMT and FLU; also baseline showed no any significant noise.

3.2 Precision

Precision of the method was studied as intra-day and inter-day variations. Intra-day variation was determined by analyzing three different concentrations 10, 15 and 20 µg/ml of AMT and of FLU, for three times within a day. Inter-day precision was assessed using same concentration of drug (mentioned above) and analyzed it for three different days, over a period of week. The results are shown in Table 4.

3.3. Accuracy

The accuracy of an analytical method is the closeness of the test result obtained by that method to true value. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay. Accuracy studies were performed by standard addition method at the 80, 100 and 120% levels as stated in ICH Guideline. The results are shown in Table 5.

3.4. Limit of detection (LOD) and limit of quantization (LOQ)

The LOD and LOQ were separately determined based on the calibration curves. The standard deviation of the y-intercepts and slope of the regression lines were used. Results of LOD and LOQ are given in Table 6.

3.5 Robustness

The robustness study was done by making small changes in optimized method parameters like change in mobile phase ratio, change in flow rate and change in column temperature. There is no significant impact on retention time and tailing factor.

3.6. Ruggedness

The ruggedness study was done by the two analysts. The % RSD of analyst-I was 0.14 for AMT and 0.26 for FLU and for analyst-II was 0.10 for AMT 0.15 for FLU.

4. Results and Discussion

The proposed HPLC method is simple, economic, accurate and reproducible and useful for simultaneous determination of AMT and FLU in combined tablet dosage form. The results of assay show the good agreement with label claim. Method was validated as per the ICH guidelines. The recoveries of drug were determined at 80, 100 and 120% level. The recovery of AMT ranges from 98.52-100.01 % and recovery of FLU ranges from 99.80-100.01, which shows the accuracy of proposed method. Inter-day and Intra-day precision was determined by analyzing the drug sample, at three different concentrations level. The intra-day and Inter-day results are presented in the form of % RSD, which is below 2.0; shows the high precision of proposed method. System suitability parameter for the proposed method was studied and verifies that the resolution and reproducibility of the chromatographic system is adequate for the analysis.

5. Conclusion

On the basis of results of assay and Validation Parameters it was conclude that proposed method was simple, fast, accurate, and precise for simultaneous estimation of AMT and FLU in combined capsule dosage form and can be applied for the routine estimation of AMT and FLU capsule dosage form.

Table. 1: Results of Linearity studies

Parameters	**AMT**	**FLU**
Linearity range	2.5 – 20 µg/ml	2.5-20 µg/ml
Coefficient of correlation (r)	0.9994	0.9995
Regression equation	Y=34360 x+1038	Y=43550x +2553
Intercept (A)	1038	2553
Slope(B)	34360	43550

Table. 2:Assay of Bulk sample.

Drug	**Amount taken (µg/ml)**	**Amount found (µg/ml) ± S.D. (n=6)**	**Amount found [%] ± S.D (n=6)**	**% RSD**
AMT	10	10.00 ± 0.017	100.03 ± 0.17	0.17
FLU	10	9.99 ± 0.022	99.99 ±0.22	0.22

n= number of repetitions

Table. 3:Assay of Capsule Formulation.

Drug	**Label claim (mg/cap**	**Amount Found (mg/ cap) ± S.D. (n=5)**	**% label claim**	**% RSD**
AMT	250 mg/cap	250.29 ± 0.3829	100.11 ± 0.1531	0.15
FLU	250 mg/cap	250.05 ± 0.2897	100.02 ± 0.1159	0.11

n= number of repetitions

Table. 4: Results from intra-day and inter-day precision

Conc. In (µg/ml)		**Intra-day**				**Inter-day**			
		Concentration found in (µg/ml ± SD (n=3)		**%RSD**		**Concentration found in (µg/ml) ± SD (n=3)**		**%RSD**	
AMT	FLU	AMT	FLU	AMT	FLU	AMT	FLU	AMT	FLU
10	10	10.03 ±648.86	10.03 ±480.4	0.19	0.11	10.01 ±495.0	9.94± 1466.7	0.14	0.34
15	15	15.01 ±3938.0	14.99 ±2600	1.14	0.59	14.99 ±999.6	15.00±2036 .2	0.19	0.31
20	20	19.99 ±955.95	19.96 ±2861	0.29	0.65	19.96 ±1233	19.99 ±5705	0.18	0.65

n= number of repetitions.

Table. 5: Recovery studies for AMT and FLU.

Drug	Amount Added (µg /ml)	Amount Recovered (µg/ ml) ± SD (n=3)	%Recovered	%RSD
AMT	0	10.00 ±0.008	100.01	0.08
	8	7.96 ± 0.05	99.52	0.62
	10	9.99 ± 0.01	99.90	0.10
	12	11.99 ± 0.05	99.90	0.45
FLU	0	10.0 ±0.006	100.01	0.06
	8	7.98 ±0.04	99.80	0.45
	10	10.00 ±0.01	100.04	0.14
	12	11.99 ±0.01	99.94	0.05

n= number of repetitions.

Table. 6: System suitability Parameters

Parameters	AMT	FLU
Retention Time (RT)	2.37	4.80
Capacity Factor (K)	0.31	0.44
Theoretical Plate (N)	344658	436581.9
Tailing Factor (T)	1.32	1.53
Limit of Detection (LOD)	0.05	0.08
Limit of Quantification(LOQ)	0.16	0.25

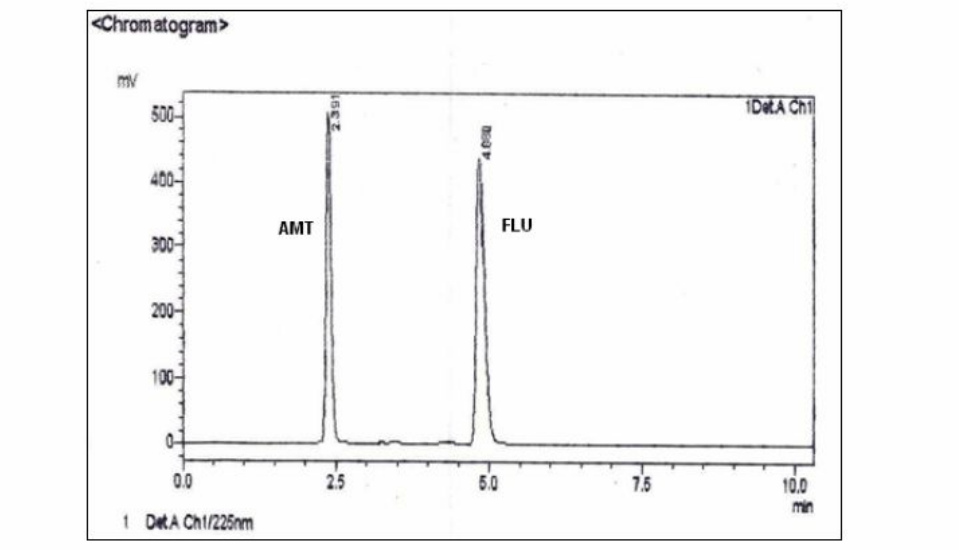

Fig.1. Chromatogram of Bulk Sample. AMT (RT=2.41 min) and FLU (RT=4.72 min)

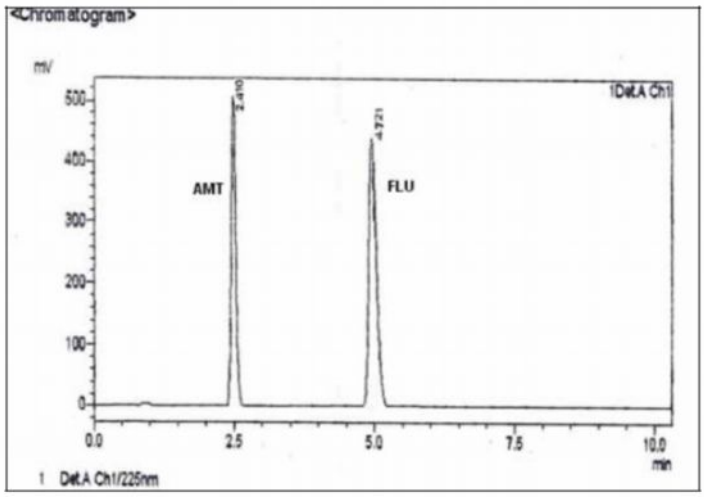

Fig. 2. Chromatogram of capsule solution. AMT (RT=2.39 min) and FLU (RT=4.68 min)

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