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Semi-automated Home-made HPLC-UV System for determination of Amoxicillin Trihydrate (AMO) in Antibiotic Drugs

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Abstract: An accurate, precise and sensitive HPLC assay was developed for the determination of Amoxicillin in Oral dosage form, to compare the bioavailability of two Amoxicillin Trihydrate (AMO) capsule (500mg) from Brazil formulations and Amoxicillin Trihydrate (AMO) Analr as a test formulation. Amoxicillin concentrations were analysed by a home-made UV-HPLC System at (λ=230 nm). The separation was achieved using the Ion Pac zorbax 300- SCX Agilent Column; 5µm, 4.6×250 mm. The mobile phase consisted of a Ammonium acetate (20 mM) + Methanol] buffer (95:5) with a pH=4.8. The study of bioequivalence between the two Amoxicillin formulations was assessed by calculating the peak height. The standard Amoxicillin and amoxicillin drug eluted at a flow rate of 1.0 ml/min. The recoveries were rang within 90.0-100% Linearity rang (0.2 -1.0) µg/ml, (n=5) with $r^2 \geq 0.9970$ and RSD ±0.505-2.672 at room temperature 25°C. The detection limit of quantification (LLOQ) was 2.830µg/ml and Lower limit of detection (LLOD) 1.047µg/ml.

Keywords: Amoxicillin trihydrate Capsules and Standardized, A Home-made HPLC-UV. .

INTRODUCTION

Amoxicillin is hydroxyphenyl-D-glycylaminopenicillanic acid trihydrate [[(2R)-Amino (4-hydroxy phenyl) acetyl]amino]-3,3 dimethyl -7-oxo-4-thia-1-azabicyclo [3.2.0] heptanes-2-carboxlic acid molecular Formula ($C_{16}H_{19}N_3O_5S.3H_2O$) and Molecular weight[1] is 419.451[1]. Antibiotics are proven medications for both human and veterinary use with broad-spectrum activity, particularly against gram Negative bacteria and positive Bacteria. Amoxicillin acts by inhibiting the synthesis of bacterial cell wall. It is soluble in water, methanol, slightly soluble in ethanol and partially insoluble in

toluene[2]. There are a several methods for quantitative determination of amoxicillin. The most interesting field of IC Chromatography application is the analysis of pharmaceutical for example, to control the quality of the pharmaceutical itself and in clinical analyses to study the effects and the evolution of the pharmaceutical in the human body[3]. The analysis of antibiotics is also important due to the wide ranging application of these drugs[4].

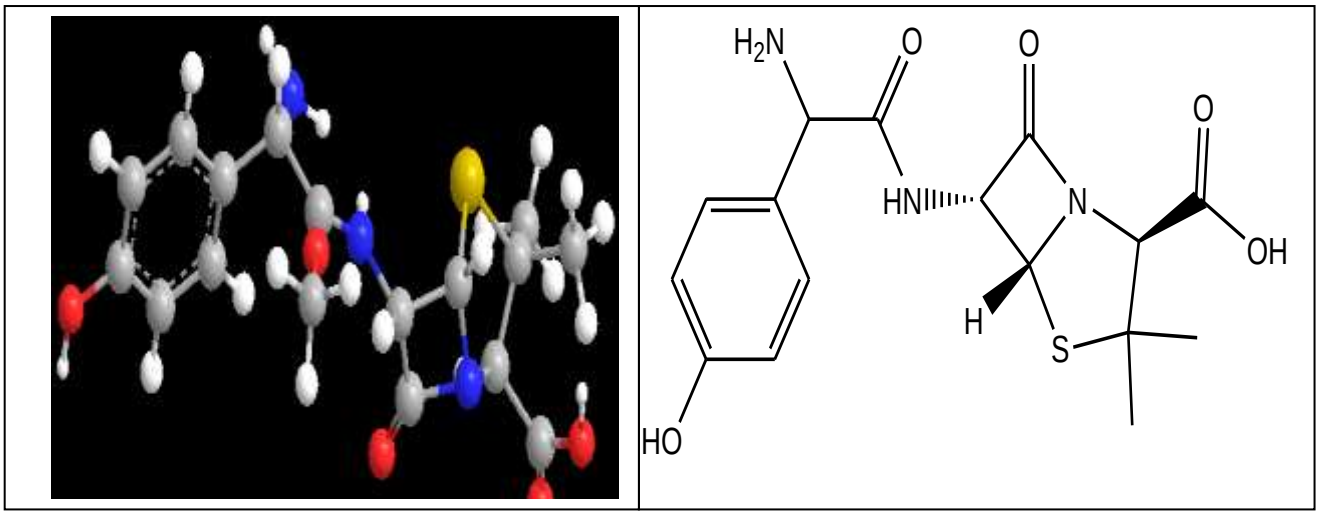

Figure 1: Structure of Amoxicillin congeners

Due to structural similarities of some impurities with amoxicillin make separation of the individual components within Amoxicillin (AMO) potentially difficult[5, 6]. However, these otherwise hydrophilic compounds can be separated by performance liquid chromatography, reversed phase- HPLC at wavelength, 230 nm that assist in accentuating the small hydrophobicity differences[7].All penicillanic acid trihydrate antibiotics contain chromophores, making UV detection with high sensitivity[8]. Amoxicillin derivatization with sulphate, hydrochloric acid or sodium salt trihydratecan be determined by changing the wavelength that depends on absorbance morality factors before sample derivatization measurements[9, 10]. The Amoxicillin are separated on a Ion pac 300-SCX Zorbax column and quantified by UV detection. This method, although effective, is an indirect detection method, which requires additional preparation time and reagents for derivatization[11].

Goal: The main aim of this study was to develop an efficient and comprehensive a home-made semi-automated HPLC - UV system for determination of Amoxicillin in antibiotic drugs.

MATERIALS AND METHOD

All solvents and reagents were of analytical grade unless indicated otherwise, and all experiments were performed with deionized water (18.2Ω-cm) resistivity[12] at 25°C.

Equipment: Chromatography experiments were carried out by a home – made -HPLC Chromatography consisting of:

- LKB Bump 2150 –HPLC, Bromma
- Ion Pac column zorbax 300- SCX Agilent Column; 5µm, 4.6×250 mm (P/N 880952-704) from USA was chosen for AMO separation.
- Metrohme Electric injection valve with 100 µL loop fitted in.
- A PD 303 UV Detector single beam (Japan) equipped with an 18 µl flow cell (Helma. UK.)
- Data logger LabJackU12 accquisions (Ocean control/ Australia).

- Personal Computer Supplied with modify software[13] programs / cvi programs UV.
- Printer (MFC-J220 Brother/ Korean).
- pH meter (Hana- Italy) .

Reagents and standards:

- Ammonium acetate HPLC grade, BDH Chem. LTD 7177- 48
- Methanol, HPLC grade, BDH M/ 405/17 LTD 116967 Cas 67-56-1
- Amoxicillin Trihydrate Capsules (Brazil) and Analar Amoxicillin powder as standard Sigma-Aldrach German.
- The Stock Standard Solution 100 µg/ml Amoxicillin Trihydrate was prepared by dissolving accurately weight 100mg of Amoxicillin trihydrate in 1000 ml methanol which was purchased from Aldrach 51/6848-LTD[14]. A working solution in the range 0.2-1.0 µg/ml was prepared by serial dilution of this stock solution with methanol. Amoxicillin Samples were prepared by powdering 10 tablets of Amoxicillin trihydrate (500 mg), 100 mg of this powder accurately weights and dissolved in 1000 ml of methanol[15].

Procedure: Under a temperature of 25 ^{o}C and pressure of 70 bar[16] all chromatography experiments were carried out by a home-made HPLC-UV chromatography system, which consisting LKB pump 2150-HPLC pumping the elunt at 1ml/min. Amoxicillin samples or standard were manually injected with Metrohm electronic injection valve fitted with 100µl loop in eluent of 20 mM ammonium acetate and Methanol (95:5) at pH 4.8[17]. Ion pac column Zorbax 300-SCX Agilent , 5µm ,4.6×250mm (p/N880952-704) was used as a separation column[18] .APD 303UVdetector single beam spectrophotometer (Japan) ,equipped with 18 µl flow cell (Helma UK) was used to measure the UV signal at 230 nm of the separated species . A data logger lab jack-Ocean control/ Australia. Personal computer and printer were handling the data of the home made system. The peak height of a symmetrical peak is corresponding to the Amoxicillin concentration of standards and sampleconcentrations[19].

Table-1: Method Parameters

Parameters	***Conditions***
Description Column	Ion Pac zorbax 300- SCX Agilent Column ; 5µm , 4.6×250 mm (P/N 880952-704)
System Suitability Requirement	USP Tailing Factor @ 5 %Peak Height 1.11 Plates 2590 - 2975
Isocratic Mobil phase	Ammonium acetate (20 mM) +Methanol (95+5) PH 4.8
Test sample	Amoxicillin diluted in the mobile phase
Detection System	UV detection
Maximum Wavelength	230 nm
Flow Rate	1.0 mL / min
Temperature	25 °C
Pressure Background	70 Bar
Run Time	14min
Injection Volume	100 µL

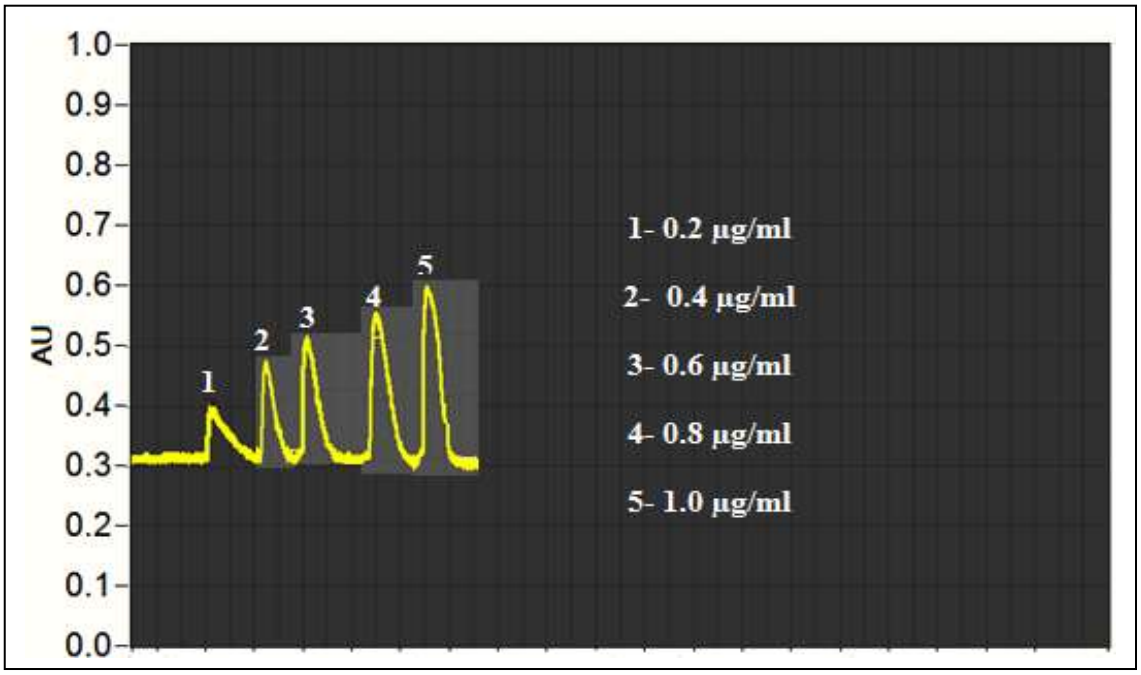

Figure 2: Chromatogram Calibration curve of Amoxicillin(AMO)

RESULT AND DISCUSSION

1. Effect of Column type, elunt Concentration and Retention Time on AMO separation: Ion Pac Zorbax 300-scx, 5 µm 4.6×250mm column was recommended as a suitable and efficient separation column for Amoxicillin[20]. Amoxicillin can be detect by using UV detector at λmax230nm with mixture of elunt consist 20 mM ammonium acetate and Methanol at 95:5 respectively , which can be freshly prepared[21] .

Figure 2 shows that the column has high efficiency to separate AMO, the linear gradient ranged between 14-15 minutes for each injection and one peak appearance in Chromatogram .The distinct peak cause of good method sensitivity to determination (AMO). But some ringing peaks refer to very small concentration of CO_2 dissolve in eluent[22].

2. Effected Column Temperature on the separation: The home- made IC system supply with Column temperature evaluating in the range 25-45^{o}C in five degree steps. As expected, increasing the column temperature decreased retention time and led to good baseline for the separation Chromatogram of the standards and samples[23]. But due to difficulties of maintaining temperature stability in the constructed home- made IC system. So 25 ^{o}C was selected to be used in future work[24].

3. Method performance (linearity, Reproducibility and Detection Limits): Under the established conditions listed in Table 1, a method of the standard calibration was used to obtain the calibration curve for Amoxicillin, by plotting the concentration versus the peak height of asymmetrical peaks. It is linear over the range (0.2-1.0) µg/ml AMO. **Table 2** lists the R_2 and slope of the curve, which are 0.9989 and 27.5 respectively.

The reproducibility of the method was estimated by injection of a 0.2, 0.4 and 0.8 µg/ml represented standard (AMO) and two commercial AMO drugs into eluent. Excellent RSD% for retention time (t_R)

and peak height were obtained as shown in **Table 2 and 3.** Lower limit of detection (LLOD) and quantitation (LLOQ), LLOD=3. 3 SD/S and LLOQ=10 SD/S are the concentrations that give the signal to noise ratio of 3:1 or 10:1 respectively. This can be detected and verified by the divided of standard deviation of response (SD) by the slope of calibration curves (S) [25, 26].By using the single-sided student's test method (at the 95% confidence limit) for five consecutive injections of 0.6 µg/ml of AMO sample and standard[27], the values of LLOD and LLOQ were 1.047 and 2.833 respectively.

Table 2: The reproducibility of peak height and t_R of AMO

Representative samples and drugs (µg mL^{-1})	**Peak height (mm)**	*** ±RSD%**	**Retention Time ($t_{R)}$ minutes**	*** ±RSD%**
0.2	**7**	**±1.672**	**7**	**±0.827**
0.4	**12**	**±1.559**	**8-9**	**±0.832**
0.8	**23**	**±0.532**	**9**	**±0.811**
5µg mL^{-1} for Drugs (1)	**103**	**±0.500**	**9-10**	**±0.808**
5µg mL^{-1} for Drugs (2)	**104**	**±0.521**	**9-10**	**± 0.850**

Table 3: Regression statistics of the proposed method with LLOD, LLOQ, Intercept and Slope

R^2	**0.9970**
Standard Error	**0.332**
Standard Error Estimate	**0.3162**
Intercept	**0.9**
Slope	**27.5**
LLOD µg mL^{-1}	**1.047**
LLOQ µg mL^{-1}	**2.830**
MDL(standard) µg mL^{-1} (SD×$t_{95\%}$) at n= 5-1	**1.090**
MDL(sample) µg mL^{-1} (SD×$t_{95\%}$) at n= 5-1	**1.5990**

[a]standard error, [b]standard error estimate

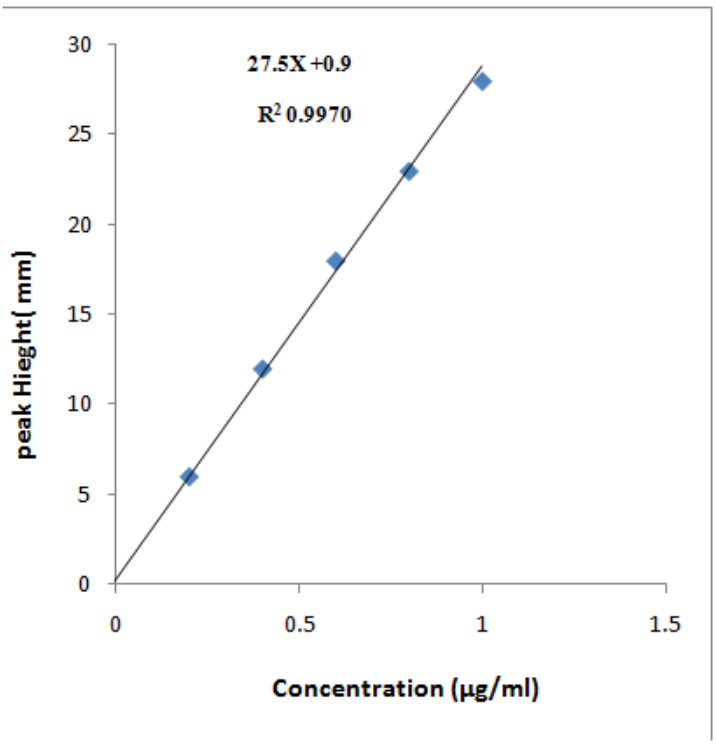

Figure 4: Standard Calibration graph of Amoxicillin (AMO) Standards

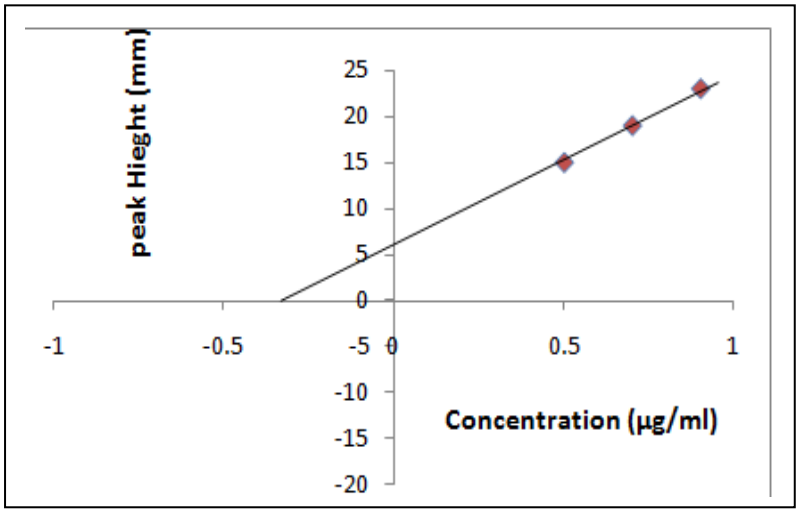

Figure5: Standard additions for amoxicillin determination

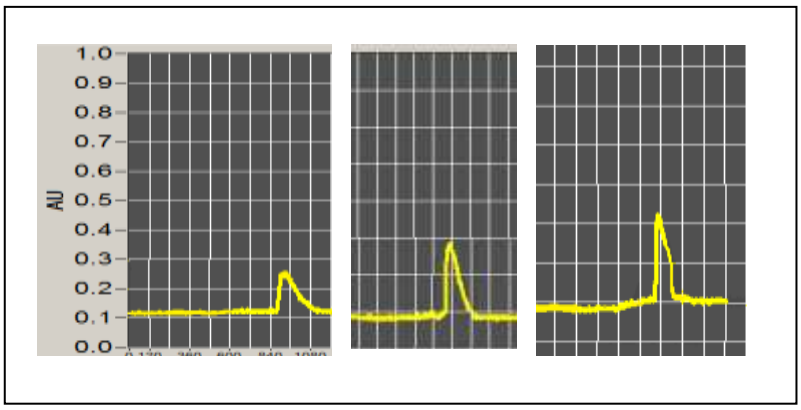

Figure 6: peaks of Standard additions Method

Accuracy: To evaluate the accuracy of the home-made HPLC-UV System. A recovery experiments were performed on three representative standards and two commercial drug samples. Standard additions method (Fig 5) was used for all of these determinations in order to avoid all the possible interferences[28, 29]. Table 4 summarized all of these studies. A good agreement between the results was obtained which clearly indicated that home-made system can be used for several applications.

Table 4: AMO recoveries obtained by home-made HPLC-UV system

Claimed Conc.(µg mL^{-1})	Found conc. (µg mL^{-1})	Recovery ± RSD
0.2	0.19	95±2.672
0.4	0.4	100±1.559
0.8	0.8	100±0.532
5µg mL^{-1} for Drugs (1)	4.9	98±0.500
5µg mL^{-1} for Drugs (2)	5.0	100±0.521

Precision: Precision of method, reported as % RSD, was estimated by measuring repeatability (intra-day assay) for five replicate injections for all concentrations of (AMO).The intermediate precision (inter-day variation) were also studied for two days using an intermediate concentration solution of (AMO).The average recoveries were in the range (95-100) which thought to be an acceptable result[30,31.]Table 5 Summarizes all of these studies.

Table5: Intra and inter-day precision and accuracy of standard analysts (n=5).

Claimed conc. (µg mL^{-1})	Intra-day		Inter-day	
	Found (µg mL^{-1})	±Recovery %RSD	Found (µg mL^{-1})	±Recovery % RSD
0.20	0.19	95 ± 1.672	0.19	95 ± 1.106
0.40	0.40	100 ± 1.559	0.38	95.0 ± 0.909
0.60	0.60	100 ± 0.878	0.60	100 ± 0.106
0.80	0.80	100 ± 0.532	0.60	100 ± 0.116
1.0	1 .0	100.0 ± 0.505	1.0	100.0 ± 0.120
µg/ml Drug (1)5	5	100 ± 0.500	5	100 ± 0.510
µg/ml Drug (2) 5	5	100 ± 0.521	5	100 ± 0.531

CONCLUSION

This work described a home- made semi –automated HPLC System equipped with UV detector for Amoxicillin determination in pharmaceutical drugs. This developed method offer simple, inexpensive and needs only a very small volume of the sample and using a UV detector makes this system very specific due to one peak in the chromatogram. In this application there is no need for high sensitivity since the pharmaceutical drugs of AMO have a very high concentration.

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