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Research Article

RP-HPLC METHOD VALIDATION FOR THE DETERMINATION OF AMPICILLIN IN IRAQI HEALTHY VOLUNTEERS SERUM

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ABSTRACT

RP-HPLC reversed-phase high performance liquid chromatographic method with UV detection was developed and validated for separation and determination of ampicillin in Iraqi healthy volunteers serum.. Mobile phase consisting of a mixture of deionized water acidified with acetic acid (0.1 %) and acetonitrile as ratio (80: 20 v/v) was delivered at a flow rate of 1.0 ml.min^{-1}. The column was Phenomenex C-18 (50 × 4.6 mm I.D) and 3 µm particle size. The eluent was monitored by UV detection at 254 nm. Analysis was performed at room temperature (~25 ^{o}C) and the total run time was 10 min. Analytical parameters linearity, accuracy, precision and specificity were determined by validation procedure and found to be satisfactory. Calibration curve was linear over the concentration range of 0.02 – 15 µg/ mL with an R^2 of (0.9994). The limits of detection (LOD) and quantification (LOQ) were 0.02 and 0.066 µg/ mL, respectively. Overall, the developed method was found to be simple, rapid, precise and accurate for quality control of ampicillin in volunteers serum.

Keywords: Ampicillin, HPLC, Volunteers, Validated.

INTRODUCTION

Ampicillin is an antibiotic, an individual from the penicillin's group of antimicrobials[1], it has been discovery first in 1961 A.D[2]. Ampicillin is a sparingly soluble in water, white crystalline, acid stable, the active group is β-Lactam[3]. The ampicillin antibiotic consists of 6-aminopenicillanic acid (6-APA)[4], structure of the ampicillin is shown in Figure (1)[5]:

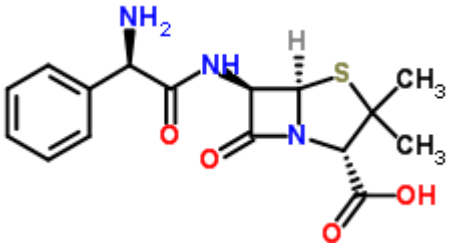

Fig. 1: structure of the ampicillin

Ampicillin is a common antibiotic that is effective against a wide assortment of Gram negative and Gram positive organisms[6]. It is semi-synthetic to penicillin, it has a wide range spectrum, impervious to the action activity of the gastric. The ampicillin is bactericide, because it represses the biosynthesis of the cellular wall of the sensitive bacteria[7]. Serum concentrations of an agent reflect its absorption, distribution, metabolism, and elimination, as well as the magnitude of the dosing regimen[8]. Determination of drugs in more complex media such as serum is needed the most suitable quantification method must be chosen. It should have a high sensitivity, specificity and reproducibility, and should be capable of measuring a wide range of concentrations of this antibiotic, besides its low

cost[9]. A few analytical techniques have been described for assay of ampicillin in pharmaceutical formulations or in blood samples using spectrophotometric methods and in biological fluids of animals including colorimetric[10]. These techniques, although still applied routinely in numerous research laboratories, are considered time-consuming and of restricted application[11]. Many methods are recorded for the determination of ampicillin[12-17]. Chromatographic analysis, in specifically high performance liquid chromatography (HPLC), is the most utilized strategy these days for determination of ampicillin due to its specificity, sensitivity, efficiency and reproducibility[18]. There are a few detection conditions related with HPLC for the quantification of ampicillin including spectrophotometry (HPLC-UV), fluorescence, and mass spectrometry (HPLC-MS)[19]. The use of high performance liquid chromatography (HPLC) as a sensitive and specific method for the analysis of ampicillin[20]. The method developed in the present work is a sensitive analytical method based on a highly specific and selective chromatographic. The goal of this method is to separation and determination of ampicillin in sera healthy volunteers.

MATERIALS AND METHODS

Chemicals

Ampicillin standard as pure powder, were generous gift from Sammara Drug Industries (SDI) Iraq. Ampicillin capsule as administrate dose for healthy volunteers were obtained from local market. Methanol and Acetonitrile (HPLC-grade) was from BDH. Potassium hydrogen phosphate (K_2HPO_4) as buffer was from BDH.

Apparatus

HPLC (Shimadzu LC – 20 A, Japan), Sartorius balance (Germany), Ultra sonic bath (Karl Kolb, Germany), Shaking water bath (Taiwan) and oven (Memmert, Germany) were used through this study.

Study design and volunteers

The study protocol was approved by the Ministry of Science and Technology, Department of Materials Research (Baghdad- Iraq). The written informed consent was obtained from all volunteers prior to study enrolment. Eleven healthy males (mean age = 35 ± 8.3 years) and nine healthy females (mean age = 35.25 ± 9.1 years) participated in this study. No enrolled volunteers had any medical problems according to drug history. Healthy volunteers were don't taken medications (including over-the-counter) neither two weeks prior to nor during the study period and all nonsmokers. Volunteers were randomly assigned to receive orally single dose 500 mg of ampicillin along with 150 mL of water. Both volunteers were managed under supervision taking after an overnight fast of at least 8 hr and subjects continued to fast for no less than 2 hr before and after dosing.

Blood sampling

To determine the serum concentration of ampicillin, 3-5 mL of whole blood was drawn from each volunteer. The time-points at which blood was collected in each case were quickly in before (0 hr) and 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 8.0 hr after administration of single oral dose in polyethylene test tubes. To obtain serum, the blood samples were then centrifuged at 4000 rpm for 15 min. at room temperature, serum was separated just after sample collection and was frozen store for subsequent assessment.

Analytical assays

Serum samples were analyzed for ampicillin using a validated high-performance liquid chromatography (HPLC) method with UV detection at 254 nm [9]. Serum samples were defrosted at room temperature. Sample preparation was done by liquid phase extraction with 0.2 ml of a mixture of methanol and (0.2 ml) of serum. Supernatant layer was separated pre-concentrated and 20 µl of sample were injected to HPLC analysis under the optimum separation conditions. Mobile phase consisting of a mixture of deionized water acidified with acetic acid (0.1 %) and Acetonitrile as ratio (80: 20 v/v) was delivered at a flow rate of 1.0 $ml.min^{-1}$ with UV detection at 254 nm. The column was Phenomenex C-18 (50 × 4.6 mm I.D) and 3 µm particle size . Analysis was performed at room temperature (~25 ^{o}C) and the total run time was 10 min.

RESULTS AND DISCUSSION

Preparation of calibration graph and linearity study

For determining the linearity, a series of solutions with a different standard ampicillin concentration range of (0.02 – 15.0 µg/mL) were prepared by simple dilution of stock solutions. The calibration graphs were obtained by plotting the area under peak versus known concentrations in µg/mL.

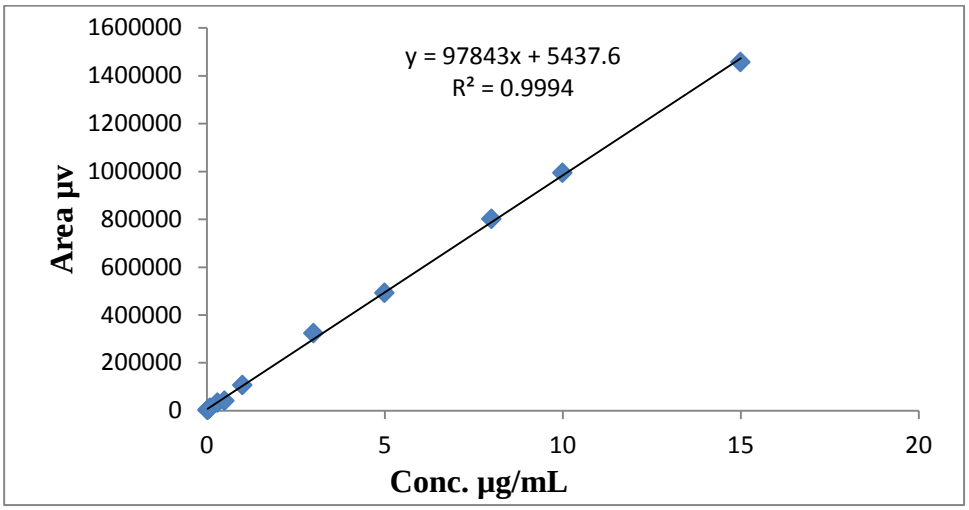

Fig. 2: Calibration graph of ampicillin

Figure 2, show the calibration graph plots of the ampicillin and the obtained results were tabulated in Table (1), which show that the values of t_{cal} are larger than t_{tab} values. The methods were linear with an R^2 of (0.9994) indicating that there is a strong correlation between the variation of concentration and response. Linearity was determined by the regression analysis.

Table 1: Statistical calculations for calibration graph of ampicillin

Statistical factors	Value
Linear equation	y = 97843x + 5437.6
Slope (m)	97843
Intercept	5437.6
Correlation coefficient "R^2"	0.9994
Percentage linearity (R^2 %)	99.94
Correlation coefficient (r)	0.9997
Standard error of intercept	4870.18
Standard deviation of intercept	16152.56
Relative standard deviation "R.S.D."	0.250
Linearity range in µg/mL	0.02 -15.0
Limit of detection "LOD" µg/mL	0.02
Limit of quantification "LOQ" µg/mL	0.066
Calculated (t) values $t_{cal.} = \frac{/r/\sqrt{n-2}}{\sqrt{1-r^2}}$	122.46 >>> 2.26

Separation and determination of ampicillin

The optimum separation conditions obtained were applied to determine the ampicillin as standard and in serum. The method exhibited good specificity and selectivity which were assessed by the retention times of ampicillin standard and for ampicillin in subject as shown in the Figure 3. The reproducibility of sample retention time compared with standard was calculated according to recovery 101.24 % .

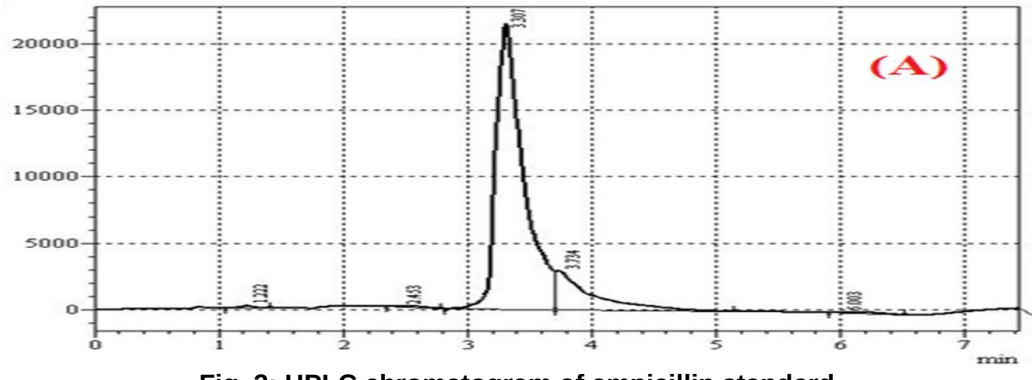

Fig. 3: HPLC chromatogram of ampicillin standard

The reproducibility (Rep.) of sample retention time compared with standard was calculated according to following equation; Rep. = (retention of sample / retention of standard) × 100
Rep. = (3.348 / 3.307) × 100 = 101.24 %
Figure 4, Represents the blank serum of healthy volunteers which not subjected to ampicillin, show no peak retention at 3.34 min.

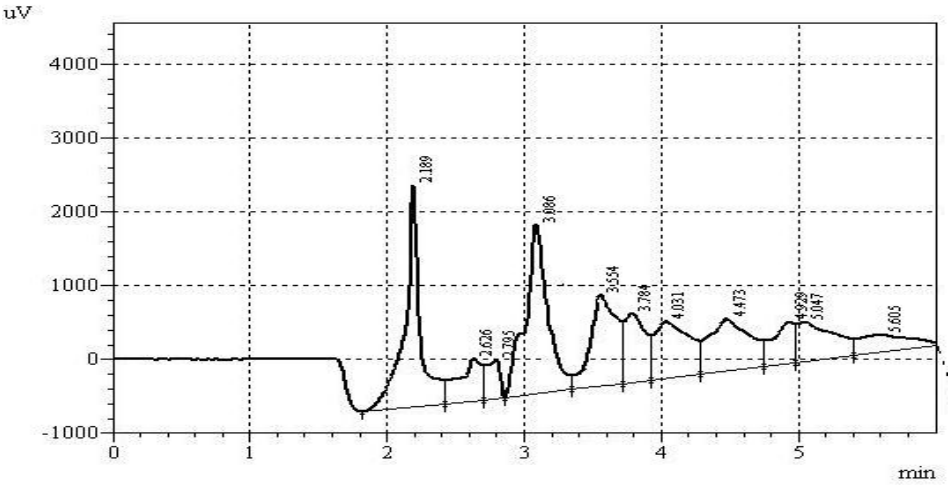

Fig. 4: HPLC chromatogram of blank serum healthy volunteers

To find the ampicillin concentrations in all subjects sera, the ampicillin peak area at retention time 3.34 min in figure 5 and the method recovery value were used to calculate the concentration.

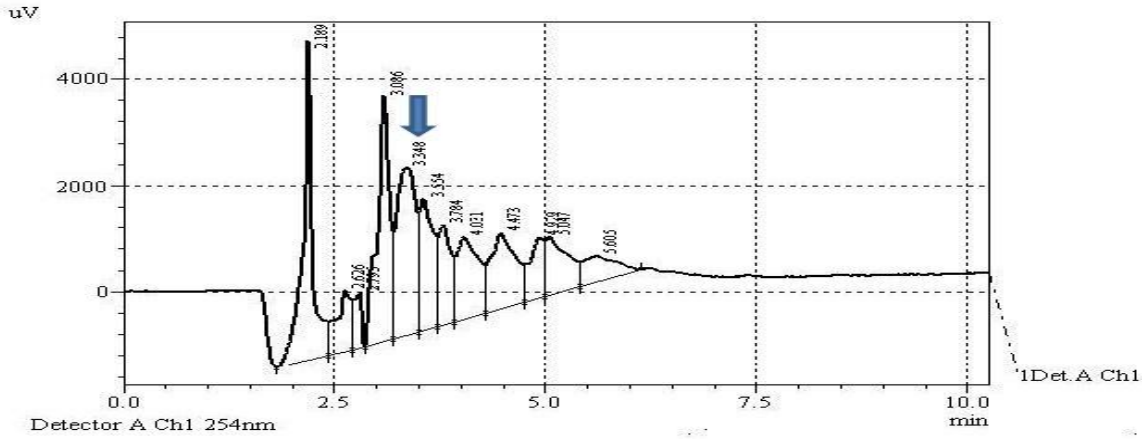

Fig. 5: HPLC chromatogram of ampicillin in serum

Accuracy and precision of proposed method

Ampicillin was determined at four different selected concentrations (0.39 – 15.0 µg/mL). The obtained results were shown in Table 2, which indicated that the proposed method for the determination of ampicillin using this method was quite satisfactory in reality with respect to the procedure and parameters calculated.

Table 2: Precision and accuracy for the determination of ampicillin in serum

Ampicillin taken µg/mL	Ampicillin found µg/mL	Recovery %	RSD% n=3
0.39	0.38	97.43	1.50
6.25	6.12	97.92	1.17
12.50	12.25	98.00	1.35
15.00	14.82	98.80	1.45

The subjects

Twenty healthy volunteers 11 males and 9 females, with average age 35.11 ± 8.3 years, To study of ampicillin determination, the characteristics population of healthy volunteers both females and males were studied. These characteristics were including the (age, sex, height and weight), of healthy volunteers with absence illness problems. As tabulated in Table 3.

Table 3: Characteristics of the volunteers (subjects)

subject	gender	Age (year)	Height (cm)	Weight (kg)
1	M	25	181	80
2	M	41	172	75
3	M	28	181	77
4	M	31	169	82
5	M	39	172	72
6	M	52	185	82
7	M	27	182	89
8	M	36	186	78
9	M	28	169	86
10	M	35	192	84
11	M	43	189	77
12	F	32	162	62
13	F	42	162	67
14	F	41	170	71
15	F	30	158	79
16	F	29	155	65
17	F	21	172	61
18	F	37	180	75
19	F	41	167	65
20	F	50	167	55

The concentration of ampicillin in serum was decreased during a time from (0 – 8 hr.) as shown in table 4. The obtained results revealed that there is significant difference in ampicillin concentration between males and females, as shown in figures 6 and 7.

Table 4: Variation of ampicillin concentration (µg/mL)

No.	Subj.	Time (hr.)							
		0	0.5	1.0	2.0	3.0	4.0	6.0	8.0
1	M	0	5.23	7.24	4.32	2.04	1.98	0.64	0.174
2	M	0	5.88	7.94	4.02	2.39	1.74	0.72	0.211
3	M	0	5.04	7.61	3.55	2.33	1.63	0.67	0.200
4	M	0	6.02	7.11	3.71	2.87	1.65	0.58	0.187
5	M	0	6.91	6.74	4.11	2.33	1.98	0.77	0.127
6	M	0	5.67	8.01	3.12	3.21	1.68	0.66	0.101
7	M	0	6.22	7.23	3.94	2.49	1.8	0.55	0.211
8	M	0	6.24	6.77	4.08	2.88	1.80	0.60	0.176
9	M	0	5.64	7.58	3.87	3.51	1.88	0.67	0.199
10	M	0	6.12	6.97	4.02	2.48	2.27	0.55	0.192
11	M	0	6.37	7.91	3.49	2.84	1.66	0.51	0.184
12	F	0	6.54	7.54	3.98	2.54	1.64	0.58	0.198
13	F	0	5.67	6.94	4.08	2.91	1.66	0.54	0.176
14	F	0	5.41	7.09	3.87	3.01	2.01	0.65	0.111
15	F	0	5.62	6.79	4.44	2.88	1.79	0.59	0.165
16	F	0	5.18	6.04	3.49	2.67	1.77	0.59	0.165
17	F	0	4.99	5.67	3.64	3.04	1.95	0.637	0.138
18	F	0	6.00	7.44	3.94	2.67	2.01	0.53	0.198
19	F	0	5.41	7.88	4.27	2.33	1.79	0.68	0.199
20	F	0	4.59	6.45	4.14	2.66	1.57	0.55	0.188

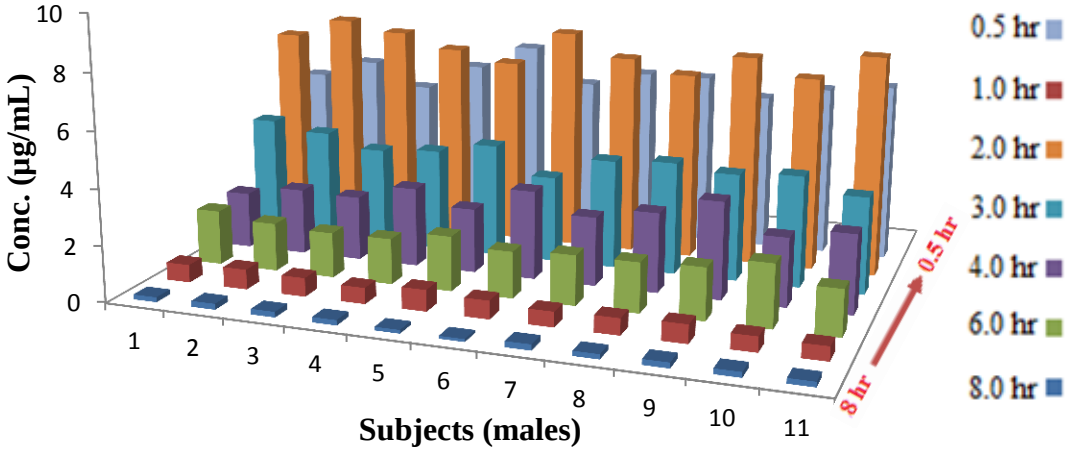

Fig. 6: Ampicillin concentrations of healthy males from 0.5-8 hr

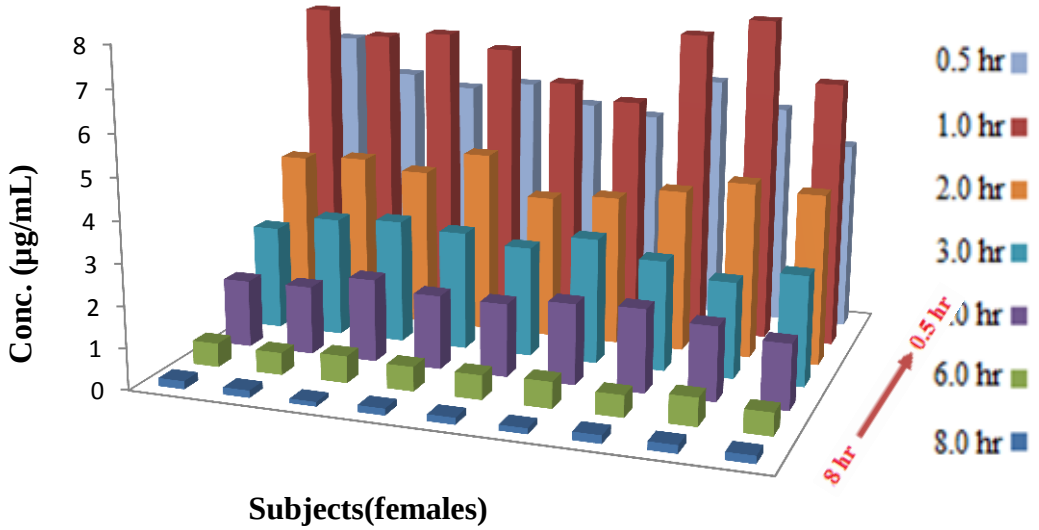

Fig. 7: Ampicillin concentrations of healthy females from 0.5 - 8 hr

CONCLUSION

The importance of this study is to separation and determination of ampicillin in serum of Iraqi healthy volunteers. The developed analytical method is simple, specific, accurate and precise for determination of ampicillin as standard and in serum. The accuracy of method was validated by mean percentage recovery which was found to be in the acceptable range.

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